Borneo Journal of Pharmacy
Vol 5 Issue 4 November 2022
Page 375 – 383

http://journal.umpr.ac.id/index.php/bjop/article/view/3683
DOI: https://doi.org/10.33084/bjop.v5i4.3683
e-ISSN: 2621-4814

Research Article

Hand Sanitizer Gel Formulation with Laccase Enzyme as an Antibacterial Against *Staphylococcus aureus* and *Escherichia coli*

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Keywords:
Active compound
Antibacterial
Hand sanitizer
Laccase enzyme
Pathogenic bacteria

Abstract

Laccase enzymes have been used widely in industrial fields such as textile, pulp, paper, food, cosmetic, and pharmaceutical industries. Laccase is used in toothpaste, mouthwash, deodorants, and soaps in personal care products. Previously, laccase enzymes had never been used for formulating hand sanitizer gel. This study aimed to determine the effect of the laccase enzyme on the physicochemical properties and the antibacterial potential of the hand sanitizer gel against pathogenic bacteria. Laccase enzyme was produced through fermentation using the fungus *Trametes hirsuta* EDN 082 with an activity of 0.032 U/mL. Hand sanitizer gel was made with the addition of laccase enzyme with varying concentrations of 4, 7, and 10% (v/v). The physicochemical test included organoleptic tests, pH evaluation, gel spreadability, and viscosity. The antibacterial was tested by the palm swab method. The gel physicochemical characteristics showed that the more laccase enzyme added, the more yellow the color produced, the less thick the shape, the wider the gel spreadability, and the lower the viscosity. The obtained pH ranged from 7.4 to 7.6. The best formulation of the hand sanitizer gel was achieved with the addition of a 7% (v/v) laccase enzyme. This formulation can reduce the number of bacteria colonies of *Staphylococcus aureus* and *Escherichia coli* on the palms with effectiveness above 95%. The laccase enzyme can be used as an active ingredient and antibacterial agent in the formulation of hand sanitizers.

Received: June 28th, 2022
1st Revised: September 19th, 2022
Accepted: October 9th, 2022
Published: November 30th, 2022

INTRODUCTION

Hand sanitizer is a practical and effective hand-washing product to inhibit the growth of bacteria such as *Staphylococcus aureus* and *Escherichia coli*. Hand sanitizer is a simple product because it is easy to use when soap and water are unavailable[1,2]. Meanwhile, hand sanitizer is selected for use because it effectively kills bacteria in a relatively fast time[3]. Hand sanitizer with an alcohol concentration of 60-70% inhibits the growth of bacteria on the hands for 1-12 minutes[4]. Alcohol inhibits bacterial growth by denaturing and coagulating bacterial cells[5].

Excessive or continuous use of alcohol-based hand sanitizers can irritate the skin, causing a burning feeling on the skin[6]. One effort to reduce chemicals in a hand sanitizer product is to innovate alcohol-free hand sanitizer products using extracts from natural ingredients that have antibacterial properties, such as lotus seeds[7], banana stems[8], shell chitosan snails[9], Binahong leaves[10], Stevia leaves, and pineapple peel[11]. According to Ariningrum *et al.*[12], hand sanitizer with Trembesi and Stevia leaf extract can reduce the number of bacteria on the hands by up to 88%.

How to cite: Anita SH, Asishe, Syafriana V, Febriani A, Zulfiana D, Oktaviani M, et al. Hand Sanitizer Gel Formulation with Laccase Enzyme as an Antibacterial Against Staphylococcus aureus and Escherichia coli. Borneo J Pharm. 2022;5(4):375-83. doi:10.33084/bjop.v5i4.3683

In addition to extractives from natural ingredients, enzymes such as proteases, lipases, amylase, cellulases, peroxidases, and laccases have also been reported as having antibacterial properties[13]. Laccase is an enzyme with a wide range of substrates, so it is applied easily in various industrial fields such as the food, textile, pulp, cosmetic, and personal care industries[14]. Laccase is widespread in higher plants such as pear, radishes, cabbage, apples, potatoes, and asparagus[15], insects such as *Anopheles*, *Apis*, *Bombyx*, *Calliphora*, *Diploptera*, *Drosophila*, and *Lucilia*[16]; bacteria such as *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Thermus thermophilus*, and *Sinorhizobium meliloti*; and fungi such as *Trametes*, *Pleurotus*, *Lentinula*, *Pycnoporus*, *Phanerochaete*, and *Agaricus*[17]. Laccase produced by bacteria and fungi is easier to obtain because secreted into the growth medium. Laccase produced by fungi has a higher potential redox value than bacteria. The potential redox value is related to the enzyme's ability to degrade high molecular-weight substrates[18].

Laccase with 0.1-5.0 mg/L in activity produced by the fungi *Myceliophthora thermophila* and *Polyporus pinisite* can inhibit the growth of Gram-positive bacteria, *Staphylococcus epidermidis*, and Gram-negative bacteria, *Pseudomonas aeruginosa* up to 99%. The antibacterial properties of the laccase enzyme are applied potentially to detergent, disinfectant, food, beverages, or cosmetic products such as soap, shampoo, deodorant, mouthwash, and contact lens cleaning fluid[19].

The laccase enzyme from *Trametes hirsuta* has never been studied before for its use in personal care products. This study aims to determine the effect of the laccase enzyme on the physicochemical properties and the antibacterial potential of the hand sanitizer gel against pathogenic bacteria. Laccase enzyme was used at various concentrations in the hand sanitizer gel formulation and was tested for its effectiveness in inhibiting the growth of *S. aureus* and *E. coli*. The stability test of the hand sanitizer gel was carried out at room temperature (27±3°C) for four weeks.

MATERIALS AND METHODS

Materials

Oil palm empty fruit bunch (OPEFB) were taken from oil palm plantations in Cikasungka, West Java, Indonesia. Trametes hirsuta EDN 082 (NCBI GenBank accession number MT476912) was isolated from Taman Eden 100, North Sumatra, Indonesia. Potato dextrose agar, malt extract, glucose, peptone, sodium benzoate, and $CuSO_4$ were purchased from Merck, Germany. Vanillin and 2,2-azino-bis-[3-ethyl benzothiazoline-6-sulphonic acid] (ABTS) were purchased from Sigma Aldrich. Petrifilm™ Staph Express Count Plate and *E. coli* Count Plate were purchased from 3M, US. Carbopol, triethanolamine (TEA), lemon oil, and propylene glycol was purchased from PT. Palapa Muda Perkasa, West Java, Indonesia.

Methods

Laccase production and extraction

Trametes hirsuta EDN 082 was cultured on Potato Dextrose Agar (PDA) and incubated at 27–30°C for seven days. Laccase enzyme was produced according to Ningsih *et al*.[20] using OPEFB as a substrate. After ten days of incubation time, the laccase on the OPEFB substrate was extracted by mixing with 0.1 M acetate buffer (pH 4.5) at a ratio of 1 : 3 (w/v) and homogenizing using an ACE AM-11 homogenizer (Nissei, Japan) at 10,000 rpm for 10 minutes under cold condition. The extraction process was conducted according to Anita *et al*[21]. The crude laccase was filtered using a nylon syringe filter of 0.22 μm and then was analyzed for its activity[22].

Preparation of laccase hand sanitizer gel

Carbopol was weighed and dispersed into sterile distilled water. The mixture was then homogenized using a homogenizer at 150 rpm for 10 minutes. After 10 minutes, sodium benzoate, propylene glycol, laccase enzymes, lemon oil, vanillin, TEA, and remaining distilled water were added to the homogenizer. The mixture was then homogenized using a homogenizer at 150 rpm for 10 minutes. The preparation was then put into a container. Hand sanitizer gel was made in three formulations with different concentrations of the laccase enzyme as an antibacterial compound (**Table I**). The commercial hand sanitizer (F4) was used for comparison with 70% of ethanol as an active ingredient.

Table I. Formulation of laccase hand sanitizer gel.

Ingredients	Function	Concentration (%)			
		Control (F0)	F1	F2	F3
Laccase	Active compound	0	4	7	10
Carbopol 940	Gelling agent	0.5	0.5	0.5	0.5
TEA	Alkaline agent	1	1	1	1
Propylene glycol	Humectant	10	10	10	10
Sodium benzoate	Preservative	0.2	0.2	0.2	0.2
Vanillin	Enhancer	0.5	0.5	0.5	0.5
Lemon oil	Fragrance	Qs	Qs	Qs	Qs
Aquades	Solvent	Ad 100	Ad 100	Ad 100	Ad 100

Physicochemical properties of laccase hand sanitizer gel

The physicochemical properties of laccase hand sanitizer gel, including organoleptic, pH value, viscosity, and gel spreadability, were evaluated. The organoleptic test was color, shape, odor, and homogeneity. The pH value was measured using a pH meter (Metrohm, Switzerland). Spreading diameter gel was measured by applying the gel to a round glass, sandwiched between two round glasses, and subjected to a 150 g load for 1 minute. The viscosity was determined using a Viscometer (RheolabQC Anton Paar, Austria) equipped with a CC27 spindle with a constant shear rate of 26/s for 60 seconds[23].

Antibacterial activity test of laccase hand sanitizer gel

The antibacterial activity test was carried out using the palm swab method. This method was carried out by taking two swabs, washing hands with running water (control), and using hand sanitizer gel after treatment[24,25]. First, hands were washed with water without using soap for 20 seconds. The palms were wiped using sterile wet cotton buds. After that, the cotton buds were dipped into a tube containing 10 mL of sterilized distilled water for dilution. A total of 1 mL of the dilution was inoculated into Petri films *E. coli* Count Plate and Staph Express Count Plate. The Petri films were then incubated at 37°C for 48 hours. Colony growth was calculated with a colony counter. The same method was used for hand sanitizer formulas F0, F1, F2, F3, and commercial (F4). The percentage of inhibition was calculated by **Equation 1**.

$$\%inhibition = \frac{Total\ control\ colony - Total\ treatment\ colony}{Total\ control\ colony} \times 100\% \quad [1]$$

Physicochemical stability test of laccase hand sanitizer gel

Hand sanitizer preparations were stored at room temperature (27±3°C) for four weeks. The stability test, which included an organoleptic test, pH value, and spreadability, was carried out once a week, while the viscosity was carried out every two weeks.

RESULTS AND DISCUSSION

The organoleptic test was carried out to evaluate the physical appearance by observing the hand sanitizer gel's color, odor, homogeneity, and shape (consistency). The color test showed that the more laccase enzyme added, the more yellow the color of the gel produced (**Figure 1**). The gel has a lemon-like smell. The odor was produced by the fragrance used. The addition of the laccase enzyme has not affected the odor of the hand sanitizer gel. All hand sanitizer gels formulation formed homogeneous, as indicated by the absence of coarse particles and no phase separation (between laccase and gel base) in the formulated hand sanitizer gel after the application on transparent glass[23]. However, the gel became more liquid when more laccase was added. Compared with commercial hand sanitizers, F0–F3 hand sanitizers look cloudy and not transparent. According to Asngad *et al.*[8], adding glycerin can cause the hand sanitizers to become clear and transparent. Besides that, glycerin can be used as a moisturizer.

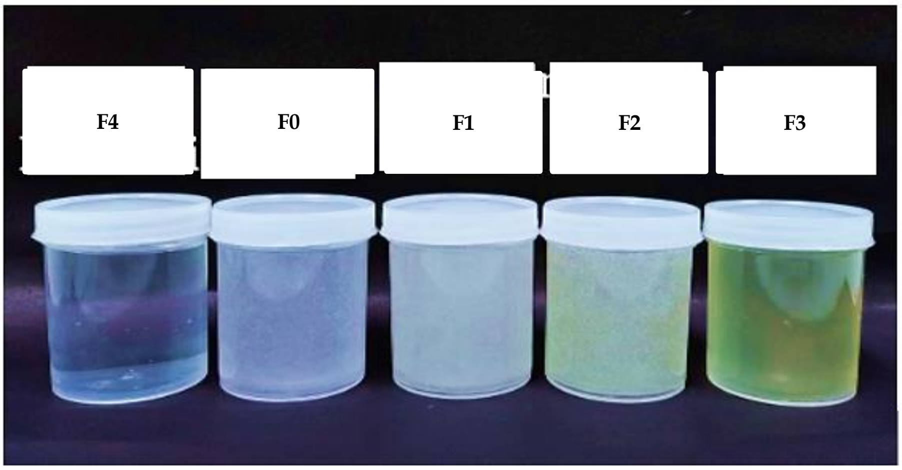

Figure 1. Laccase hand sanitizer gel, without laccase (F0), laccase 4% (F1), laccase 7% (F2), laccase 10% (F3), and commercial hand sanitizer (F4).

The pH test aims to see the safety of the formulated hand sanitizer gel so that it does not irritate the skin when applied[26]. According to the Indonesian National Standard (SNI), the pH value of topical preparations is 4.5–8[27]. The pH values of formulated hand sanitizer gels (F0–F3) were slightly higher than commercial hand sanitizers. However, the formulation F0–F3 had a pH in the skin pH range, and therefore it is safe to use. The variations of the laccase enzyme concentration in hand sanitizer had not significantly affected the pH value (**Table II**).

The viscosity test was carried out to determine the consistency and flowability of the gel formulation when applied to the skin. As shown in **Table II**, the more laccase enzyme added, the lower the formulated hand sanitizer gel viscosity. The viscosity values produced by all formulations are lower than commercial hand sanitizers. Asngad *et al*.[8] reported that hand sanitizer gel that consists of banana stem extract, alcohol, triclosan, and glycerin produces viscosity values between 520–1.250 cPs. Factors that affect the low viscosity value of formulation gel include the formulas' pH, the extract's pH, the amount of Carbopol and TEA used[8], and mixing or stirring during the formulation process[28].

The spreadability test was carried out to determine the ability of the gel to spread on the skin surface. The greater the spreadability, the easier the gel to apply to the skin's surface. If the gel is too watery, then the gel will be challenging to stick to the skin. If the gel is too thick, then the gel is difficult to apply to the skin surface[29]. The higher the concentration of the laccase enzyme used, the greater the spreadability of the preparation (**Table II**). The lower the viscosity value, the higher the spreadability[10,30].

Table II. The pH, viscosity, and spreadability values of hand sanitizer gel formulation.

Formulation	Physicochemical characteristic		
	pH	Viscosity (cPs)	Spreadability (cm)
F0	7.42 ± 0.094	1032 ± 5.65	4.79 ± 0.40
F1	7.57 ± 0.059	572.4 ± 14.70	5.04 ± 0.38
F2	7.48 ± 0.038	363.9 ± 4.10	5.06 ± 0.95
F3	7.64 ± 0.046	48.04 ± 11.08	5.23 ± 0.18
F4	6.44 ± 0.089	1223 ± 0.00	4.57 ± 0.40

The result of the antibacterial activity of laccase hand sanitizer gel can be seen in **Figure 2**. As shown in **Figure 2**, the higher the laccase concentration in the hand sanitizer formula, the greater the inhibition of bacterial growth against *S. aureus* and *E. coli*. The activity of the laccase enzyme stock used was 0.032 U/mL. The laccase enzyme activity detected in the hand sanitizer formulation with adding the laccase enzymes 4, 7, and 10% were 0.003, 0.005, and 0.013 U/mL, respectively. The higher the laccase enzyme concentration added, the higher the enzyme activity value detected in the preparation. The results revealed that the highest inhibition against *S. aureus* was shown by F2 (96.77%), while in F3, it decreased (95.63%). The effectiveness of F2 against *S. aureus* was the closest to the growth inhibition of the commercial product F4 (97.90%).

Different things happened to the growth of *E. coli*. The percentage of inhibition of *E. coli* for all concentrations from F1 to F3 was 100%, the same as that of commercial product F4. This result is different from antibacterial solid bath soap made from palm leaf, which is only able to inhibit the growth of *S. aureus*[31]. In most cases, antibacterial compounds are more resistant to Gram-negative than Gram-positive bacteria. This is because of the different composition and structure of the two bacteria's cell walls. Gram-positive bacteria have a simpler and thicker cell wall structure than Gram-negative bacteria, with a single layer that is 15-80 nm thick, low in lipid content (1–4%), and contains teichoic acid. At the same time, the Gram-negative bacteria have three-layered cell walls with a thin (10-15 nm) outer layer, a high lipid content (11–12%), and peptidoglycan located in the rigid inner layer with a small amount of about 10% dry weight and no teichoic acid[32,33]. The results of this study, which showed that *E. coli* was more sensitive than *S. aureus*, suggest that a hand sanitizer formula with laccase could more easily lyse the outer layer of Gram-negative bacteria's cell wall in the form of lipopolysaccharides. However, this hypothesis should be investigated further in future research.

These findings suggest that the laccase enzyme could be used as an active ingredient in hand sanitizers because it inhibits bacterial growth. Laccase has been shown to have the same antibacterial properties as hand sanitizers containing 60–95% alcohol[24]. Furthermore, laccase-containing hand sanitizers inhibit bacterial growth even more effectively than those containing 1.5–2% triclosan, which only suppresses bacterial growth by 62.94–64.5%[34]. Triclosan is an active substance that is generally added to antibacterial bath soaps[35].

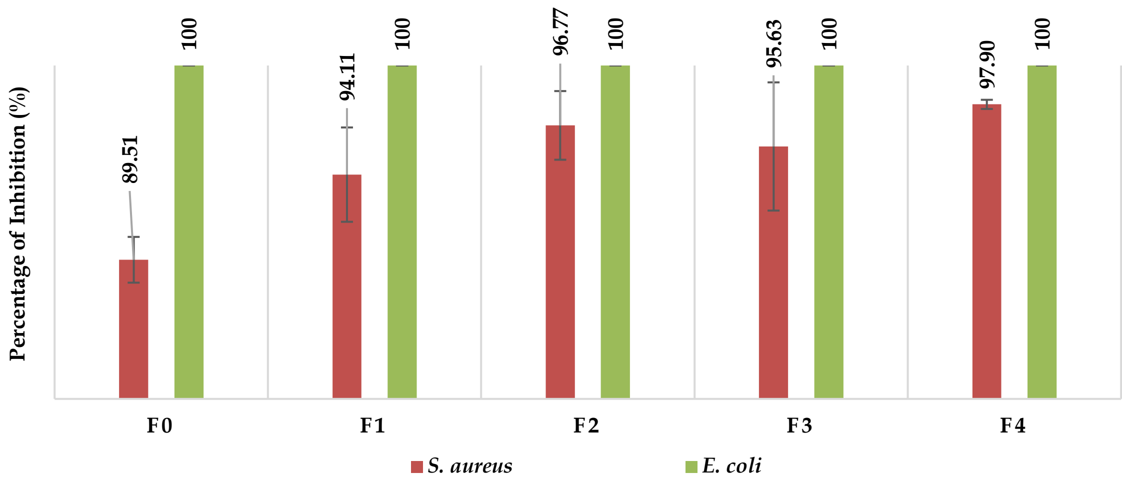

Figure 2. The percentage of inhibition of hand sanitizer gel formulation against Gram-positive-bacteria, *S. aureus*, and Gram negative-bacteria, *E. coli*.

The stability test included an organoleptic test, pH value, viscosity, and spreadability. The organoleptic test revealed no color, odor, or shape differences (**Table III**). This demonstrates that the laccase enzyme active ingredient in the hand sanitizer gel is stable after four weeks of storage. During the storage, the pH of each formula did not change significantly (from 7.42 to 7.71). Despite this, the pH value met the SNI's requirements (4.5–8). **Table III** showed no significant differences in pH changes throughout the week. During storage, F0 and F1 spreadability ranged from 3.85 to 5.37 cm. Meanwhile, F2 and F3 spreadability ranged from 3.89 to 5.83 cm (**Table III**). The viscosity of the formula is inversely proportional to its spreadability[36]. The dispersion of each formula appears to fluctuate during storage. Small changes in spreadability indicate that the formula has good dispersion stability[11].

The viscosity of each formula decreases as storage time increases until four weeks (**Figure 3**). Viscosity loss can be caused by factors such as light and humidity in the storage environment. Uncontrolled humidity in the storage room can cause the gel absorbs moisture from the air, thus lowering the gel's viscosity. Less impermeable packaging can cause the gel to absorb moisture (hygroscopic) from the outside, causing the volume of water in the gel formula to increase[37].

Table III. The physicochemical stability test of laccase hand sanitizer gel during storage.

Formula	Organoleptic characteristics			pH value	Spreadability (cm)
	Color	Odor	Shape		
Week-0					
F0	W	L	G	7.42 ± 0.094	4.79 ± 0.40
F1	W	L	G	7.57 ± 0.059	5.37 ± 0.25
F2	W	L	G	7.48 ± 0.038	5.58 ± 0.38
F3	WY	L	Lq	7.64 ± 0.046	5.10 ± 0.03
Week-1st					
F0	W	L	G	7.47 ± 0.063	5.10 ± 0.25
F1	W	L	G	7.62 ± 0.087	5.19 ± 0.29
F2	W	L	G	7.47 ± 0.051	5.23 ± 0.41
F3	WY	L	Lq	7.55 ± 0.153	5.83 ± 0.70
Week-2nd					
F0	W	L	G	7.51 ± 0.067	4.64 ± 0.57
F1	W	L	G	7.67 ± 0.036	4.60 ± 0.36
F2	W	L	G	7.46 ± 0.051	4.98 ± 0.52
F3	WY	L	Lq	7.64 ± 0.079	4.26 ± 0.47
Week-3rd					
F0	W	L	G	7.47 ± 0.071	3.91 ± 1.17
F1	W	L	G	7.50 ± 0.119	4.15 ± 0.98
F2	W	L	G	7.46 ± 0.041	3.89 ± 1.27
F3	WY	L	Lq	7.68 ± 0.023	4.81 ± 0.49
Week-4th					
F0	W	L	G	7.50 ± 0.048	4.00 ± 0.80
F1	W	L	G	7.60 ± 0.064	3.85 ± 1.24
F2	W	L	G	7.46 ± 0.056	4.14 ± 0.95
F3	WY	L	Lq	7.71 ± 0.009	4.86 ± 0.96

W: white; WY: white yellow; L: lemon; G: gel; Lq: Liquid

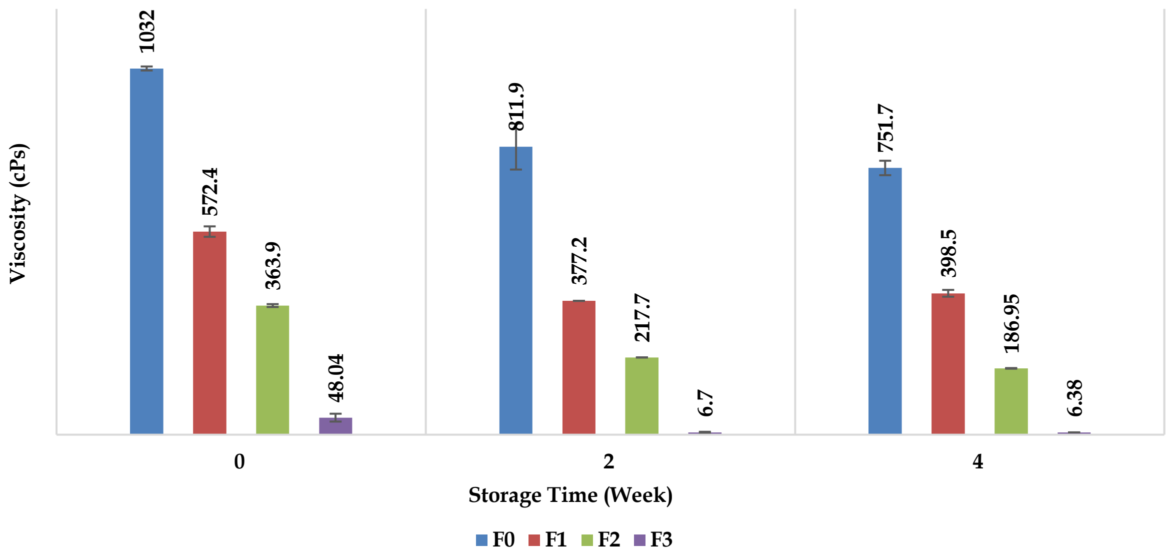

Figure 3. The viscosity of hand sanitizer gel formulation during storage time.

CONCLUSION

Laccase can be used as an active ingredient in hand sanitizer gel formulations. Variations in the concentration of laccase affect the characteristics of the hand sanitizer gel produced. Hand sanitizer gel formulation with laccase at concentrations of 4, 7, and 10% inhibited the growth of *S. aureus* to 94.11, 96.77, and 95.63%, respectively. All formulations are known to inhibit the growth of *E. coli* by 100%. Formula F2, with a laccase concentration of 7%, shows the best performance with the most significant inhibition on tested pathogenic bacteria.

ACKNOWLEDGMENT

The authors thank the Integrated Laboratory of Bioproducts (ILaB) at the National Research and National Agency (BRIN), Cibinong, Bogor, Indonesia, for the facilities and scientific and technical assistance. BRIN supported part of this research through DIPA 2021 and by Japan-ASEAN Science, Technology, and Innovation Platform (JASTIP) Network.

AUTHORS' CONTRIBUTION

Sita Heris Anita: designed the study, analyzed data, supervised the experiment, wrote, reviewed, and edited the manuscript. **Asishe**: performed research, collected data, and wrote the initial manuscript. **Vilya Syafriana**: analyzed data, wrote, reviewed, and edited the manuscript. **Amelia Febriani**: analyzed data, wrote, reviewed, and edited the manuscript. **Deni Zulfiana**: analyzed data, supervised the experiment, reviewed, and edited the manuscript. **Maulida Oktaviani**: analyzed data and supervised the experiment. **Oktan Dwi Nurhayat**: analyzed data and supervised the experiment. **Dede Heri Yuli Yanto**: analyzed data, reviewed, and edited the manuscript.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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