Bioconversion of Glycerol to Biosurfactant by Halophilic Bacteria *Halomonas elongata* BK-AG18

Mieke Alvionita[#] and Rukman Hertadi[*]

Department of Chemistry, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40135, West Java, Indonesia

[*] ***Corresponding author:***

email: rukman@chem.itb.ac.id

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Abstract: *The increasing production of biodiesel is typically followed by the increasing number of glycerol as co-product. The abundance of glycerol will cause an environmental problem since it can be used as the carbon source for bacterial growth including pathogenic bacteria. In this study, four moderate halophilic bacteria indigenous from Bledug Kuwu Mud Crater, Central Java, Indonesia were screened based on their capability to bioconvert glycerol to biosurfactant. This study found* Halomonas elongata *BK-AG18 as the potential bacterium that able to perform such bioconversion. The optimum condition for the bioconversion of glycerol into biosurfactant was attained when the bacterial inoculum was grown in the medium containing 2% (v/v) glycerol, 0.3% (w/v) urea, and 5% (w/v) NaCl at 35 °C and pH 6. The resulted biosurfactant has emulsification index (EI_{24}) about 53.6% and CMC about 275 mg/L. Preliminary structural analysis using FTIR and ^{1}H-NMR indicated that biosurfactant produced by* H. elongata *BK-AG18 was likely a glycolipid type. The biosurfactants have antibacterial activity against* Staphylococcus aureus *with a minimum inhibitory concentration of 433 mg/L. Our study thus showed that* H. elongata *BK-AG18 was the potential halophilic bacteria that can bioconvert glycerol into glycolipid type of biosurfactant with antibacterial activity.*

Keywords: *glycerol; biosurfactant; halophilic bacteria;* Halomonas elongata *BK-AG18*

■ INTRODUCTION

The limited availability of petroleum has led to the development of renewable energy sources. One of the promising fuels that can substitute the fossil fuel is biodiesel [1]. Biodiesel can be produced by base-catalyzed transesterification of triglyceride with alcohol. This reaction, however, also generates glycerol as the co-product. Thus, the increasing production of biodiesel will be followed by the increasing number of glycerol co-production that will cause some environmental problems [2]. By performing bioconversion of glycerol into bio-based materials, such as biosurfactant, is expected able to reduce the negative impact of this pollutant on the environment.

Biosurfactants are surface-active molecule synthesized by the microorganism. They are amphiphilic compounds produced on the microbial cell surface or secreted extracellularly. Glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate biosurfactants are the major classes of biosurfactants [3]. Because of their high biodegradability, biosurfactants are more environmentally friendly than the chemical surfactant [4-5]. Due to their favorable nature, biosurfactants have many potential applications in enhanced oil recovery, hydrocarbon degradation, antimicrobials, heavy metal remediation, and food industries [6-7].

The capability of some local halophilic bacteria to bioconvert glycerol into biosurfactants is interesting to be studied. This interest in using halophilic bacteria was based on the study conducted by Dhasayan et al. who found that these bacteria produced biosurfactant as their self-defense against the high saline environment [8]. In the present study, four moderate halophilic bacteria indigenous to Bledug Kuwu Mud Crater, Central Java, Indonesia have been screened. One most potential bacterium was selected, and the optimization condition of biosurfactant production was evaluated. The

[#] *Present address: Department of Chemistry, Malang State University*

physicochemical property of the biosurfactant was examined based on its activity to emulsify palm oil and also based on the decreasing of the surface tension of water at the critical micelle concentration. A study by Donio et al. noted the potential of biosurfactant produced by halophilic bacteria, *Halomonas* sp. BS4 was able to inhibit the growth of some pathogenic bacteria [9]. Therefore, in the present work, the preliminary study to evaluate the antibacterial activity of the resulted biosurfactant was also performed.

■ EXPERIMENTAL SECTION

Materials

Four moderate halophilic bacteria indigenous from Bledug Kuwu Mud Crater including *Halomonas elongata* BK-AB8, *Halomonas elongata* BK-AG18, *Halomonas meridiana* BK-AB4, and *Chromohalobacter japonicus* BK-AB18 were obtained from the collection of biochemistry laboratory of ITB [10]. Inorganic chemicals, such as NaCl, KH_2PO_4, K_2HPO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, $FeSO_4{\cdot}7H_2O$, HCl, NaOH were purchased from Merck. Organic chemicals, such as urea, glycerol, chloroform, acetone, and methanol were also purchased from Merck. Medium components, such as peptone, yeast extract, beef extract, bacto agar were purchased from Difco.

Instrumentation

UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) was used in this study to measure bacterial growth regarding optical density. Fourier transformed infrared spectrophotometer (Shimadzu IR Prestige-21, Japan) and ^1H nuclear magnetic resonance (Magritek Spinsolve, Germany) were used for the preliminary structural characterization of biosurfactant, while biosurfactant activity was measured by Du Nouy tensiometer (Thermo Fisher Scientific, USA).

Procedure

Inoculum preparation and culture medium

The inoculum was prepared by inoculating the bacteria in Luria Bertani medium containing in g/L: 100.0 NaCl, 10.0 peptone, 5.0 yeast extract and incubated under aeration of 150 rpm, at 37 °C for 24 h.

Screening of biosurfactant-producing bacteria

The Gls-Ur-MSM is the medium used for screening of biosurfactant production that composed of following components in g/L: 3.0 urea, 0.6 KH_2PO_4, 1.0 K_2HPO_4, 50.0 NaCl, 0.6 $MgSO_4{\cdot}7H_2O$, 0.01 $CaCl_2{\cdot}2H_2O$, 0.01 $FeSO_4{\cdot}7H_2O$, and 1.0 yeast extract followed by sterilization at 121 °C for 15 min. Glycerol (3% v/v) was added to the medium after sterilization. One percent (v/v) of inoculum was cultivated in the Gls-Ur-MSM under agitation rate of 150 rpm at 37 °C for 96 h. The optical density was measured at 600 nm (OD_{600}) using UV-Vis spectrophotometer, and the biosurfactant activity was determined by oil spreading test (OST). The efficiency of biosurfactant production was calculated by dividing the oil spreading diameter with OD_{600} then multiplied by 100.

Oil spreading test (OST)

The culture was centrifuged at 4 °C with the spin rate of 8000 rpm for 30 min to obtain cell-free supernatants. Forty milliliters of water was added to a petri dish (diameter of 15 cm) followed by adding 1.0 mL of palm oil. The cell-free supernatant was dropped onto the oil surface, and the oil spreading diameter was measured. This method was modified from Monira and Abdelhadi [11].

Optimization for biosurfactant production

There are five parameters considered in this study to affect the biosurfactant production, such as glycerol, urea, and NaCl concentrations, the medium pH and temperature. In order to optimize the biosurfactant production, a series of experiment was performed by changing one parameter at a time while keeping the others remain constant. Glycerol, NaCl, and urea concentrations were varied from 0–8% (v/v), 2–20% (w/v), and 0–0.8% (w/v), respectively. pH of the medium culture was adjusted to 3–10, whereas the temperature was set from 25–50 °C. After 48 h of incubation, OD_{600} and OST were measured.

Production and purification of biosurfactant

Biosurfactant dissolved in the cell-free supernatant was precipitated using acetone and then extracted with chloroform/methanol (2:1) mixture. Biosurfactant was

analyzed by thin layer chromatography (TLC) using $CHCl_3/CH_3OH/H_2O$ (65:25:4) as the mobile phase. The separated components were detected by UV light exposure [12].

Physicochemical characterization of biosurfactant

Critical micelle concentration (CMC). Different biosurfactant concentration was prepared from 50–500 mg/L, and the surface tension was measured using Du Nouy tensiometer at room temperature. The critical micelle concentration (CMC) of the biosurfactant was determined by measuring the decreasing of the surface tension of water as the function of the biosurfactant concentration [13].

Emulsification index (EI_{24}). The emulsification index was determined by adding 2.0 mL of biosurfactant at the CMC into a glass tube containing 2.0 mL of palm oil and then mixed using vortex for 2 min. After 24 h, the emulsification index at the 24th h (EI_{24}) was determined by following formula [14]:

$$EI_{24} = \frac{h_e}{h_s} \times 100\ \% \quad (1)$$

where h_e is the height of the emulsion layer, and h_s is a total height of the mixture between solution and emulsion.

Partial structural characterization of biosurfactant

Fourier transform infrared (FTIR). The functional groups of biosurfactants were analyzed using Fourier Transform Infrared (FTIR) spectrophotometry. The sample was prepared by the KBr method, and the FTIR spectrum was measured from the wave number of 900 to 4000 cm^{-1}.

^{1}H nuclear magnetic resonance (NMR). The ^{1}H-NMR analysis of the biosurfactant sample was carried out in Magritek Spinsolve 43 MHz spectrometer at 25 °C. The sample was prepared by dissolving 20 mg of biosurfactants in 500 µL chloroform.

Antibacterial assay

The antibacterial activity of the biosurfactant was examined against the growth of *Escherichia coli* and *Staphylococcus aureus*. 1000 mg/L of purified biosurfactant was added to the medium containing the following components in g/L: 1.0 NaCl, 1.0 peptone, and 0.5 beef extract. As much as, 1% (v/v) of the target bacterial cultures were transferred into the medium and then it was incubated in a shaking incubator with the agitation rate of 150 rpm, at 37 °C, overnight. The bacterial growth was monitored in terms of OD_{600} that measured by UV-Vis spectrophotometer. The result was compared to OD_{600} of each bacterial inoculum in the same medium without the addition of biosurfactant. The minimum inhibitory concentration was determined by varying biosurfactant concentration from 200 to 1000 mg/L.

■ RESULTS AND DISCUSSION

In this study, glycerol was used as a carbon source for biosurfactant production. Several studies have reported the success of glycerol bioconversion by certain bacteria to biosurfactant. *Pseudomonas aeruginosa* has been reported to have the capability to convert glycerol into rhamnolipid [15], while *Bacillus subtilis* has been shown to have the capability to metabolize glycerol to become surfactin, a lipopeptide-type of biosurfactant [16]. In addition, bioconversion of glycerol into lipid mannosylerythritol was also successfully performed by *Pseudozyma antarctica* [17].

It is intriguing to know whether or not halophilic bacteria from our local natural source, Bledug Kuwu salty mud crater, Central Jawa, Indonesia, that have been previously isolated and identified as *H. elongata* BK-AB8, *H. elongata* BK-AG18, *H. meridiana* BK-AB4, and *Chromohalobacter japonicus* BK-AB18 also exhibit its capability to bioconvert glycerol into certain type of biosurfactant. In this study, the potential screening of those bacteria to bioconvert glycerol into biosurfactant was conducted by oil spreading test. The potential bacteria was then optimized for its capacity to produce biosurfactant by varying medium compositions and growth conditions. The resulted biosurfactant was characterized by its physicochemical properties and studied its potential as an antibacterial agent.

Screening of Biosurfactant-Producing Bacteria

Each bacterium used in this study was grown in Gls-Ur-MSM medium containing 3% (v/v) glycerol for

Table 1. The efficiency of biosurfactant production

Bacteria	Oil spreading diameter (cm)	Optical density (OD)	Efficiency (%)
C. japonicus BK-AB18	0.4	1.128	35
H. meridiana BK-AB4	1.0	1.655	60
H. elongata BK-AB8	0.5	1.839	27
H. elongata BK-AG18	0.3	0.473	63

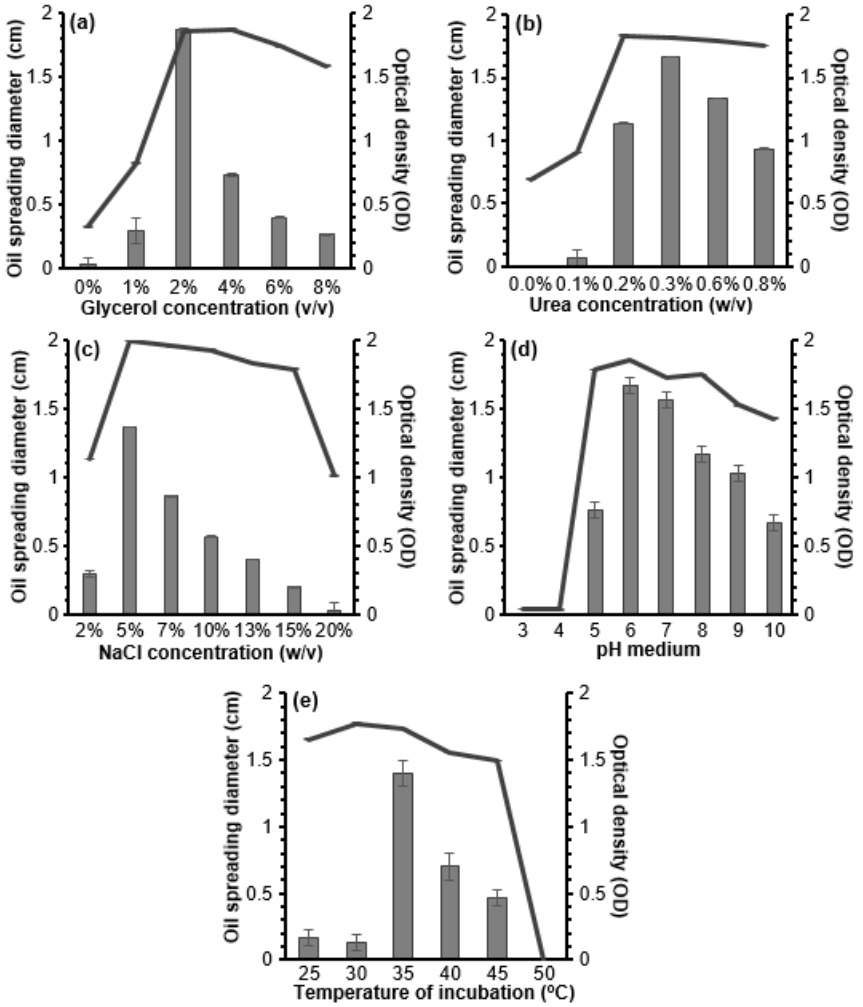

Fig 1. Optimum condition of biosurfactant production by *H. elongata* BK-AG18 in various (a) glycerol concentration (b) urea concentration (c) NaCl concentration (d) pH medium (e) temperature of incubation. The line represents optical density (OD), and histogram represents oil spreading diameter

96 h. After separating supernatant and pellet cells by centrifugation, the presence of biosurfactant in each supernatant was examined by oil spreading test (OST). In this test, a supernatant was dropped onto palm oil layer that previously added to cover a water surface in a petri dish. If the supernatant is containing biosurfactant, the oil layer will be emulsified and creating the clear zone on this layer. The length of the clear zone diameter, the higher of the biosurfactant activity. In this experiment, all screened bacteria exhibited potential to bioconvert glycerol to biosurfactant as shown by the positive result of OST (Table 1). However, all of the bacterial strains

exhibited different efficiency in converting glycerol to biosurfactant. This efficiency value was calculated by dividing the clear zone diameter with the value of OD_{600} of bacterial cells. The result showed that *H. elongata* BK-AG18 gave the highest efficiency to produce biosurfactant (Table 1). The biosurfactant resulted by this bacterial strain, therefore, was further studied in the next work.

Optimum Condition for Biosurfactant Production by *H. elongata* BK-AG18

Many factors affect the bacterial growth and biosurfactant production such as carbon and nitrogen sources, and the growth conditions, such as temperature, pH, pressure, etc. [18-19]. Therefore, those factors should be optimized to obtain the maximum production of biosurfactant.

After varying glycerol concentrations as a carbon source in the production medium, it appeared that the optimum glycerol concentration for biosurfactant production was 2% (v/v) (Fig. 1a), in which at this concentration, the clear zone diameter evolved from OST reached about 1.9 cm. At higher concentration of glycerol, the diameter of clear zones from OST was getting shorter, e.g., at 8% (v/v) of glycerol, the diameter of clear zones averagely only about 0.4 cm. Since the bacterial growth from 2 to 8% (v/v) of glycerol was relatively similar as indicated by OD_{600} values, it is suggested that higher glycerol concentration was likely to inhibit bioconversion to biosurfactant.

In order to optimize the concentration of nitrogen source in the production medium, variations of urea concentration ranging from 0 to 0.8% (w/v) were made. After comparing the clear zone diameter from OST result, it was revealed that the highest level of bacterial growth and biosurfactant production was exhibited by the medium containing 3% (w/v) urea. Urea concentrations that higher than 0.3% (w/v) tend to decrease the level of biosurfactant production and bacterial growth. According to Maneerat, the production of surface-active compounds often occurred when the nitrogen source is limited in the culture medium [20].

The effect of NaCl concentration was also considered since this study used halophilic bacteria. After

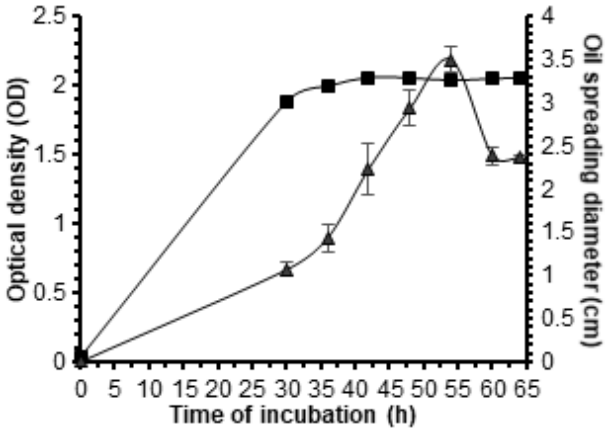

Fig 2. Growth profile and biosurfactant production by *H. elongata* BK-AG18. (▪) represents optical density (OD) while (▲) represents oil spreading diameter

growing the bacteria in the production medium containing a different concentration of NaCl and testing the resulting biosurfactant, it was found that the optimum NaCl concentration for the biosurfactant production was 5% (w/v) (Fig. 1c). The effect of NaCl concentration was similar to that of glycerol since both were not affecting the bacterial growth significantly but tightly controlling the metabolic pathway towards biosurfactant productions.

Besides optimizing the medium compositions, the effect of the other factors, such as pH and temperature were also evaluated. Varying pH and temperature of medium gave different effect to the profile of growth and biosurfactant production (Fig. 1d and 1e). As shown in Fig. 1d, at pH 3 and pH 4, no growth was observed, and consequently, no oil spreading activity was noticed. Meanwhile within pH 7–10, gradually decreasing of biosurfactant production was observed. The highest oil spreading diameter occurred at the pH 6, so this pH was used to produce biosurfactant in the next production. Different from the effect of the pH medium, the temperature effect was highly affecting the growth and biosurfactant production. As depicted in Fig. 1e, the optimum temperature for biosurfactant production occurred when the bacteria was growing at 35 °C. When the temperature increased to 40 and 45 °C, the biosurfactant production was sharply decreasing, and no biosurfactant activity was observed when the bacteria

were grown at 50 °C. Therefore, it is likely that enzymes involved in the biosurfactant production were more tolerance towards pH change than temperature change.

Based on the results above, the best biosurfactant production can be achieved when *H. elongata* BK-AG18 was grown in the medium containing 2% (v/v) glycerol, 0.3% (w/v) urea, and 5% (w/v) NaCl, pH 6 and incubating at 35 °C. After using all of these optimum conditions, the highest biosurfactant production was observed at 54th h of cultivation (Fig. 2). At this time, *H. elongata* BK-AG18 was in the stationary phase wherein the nutrients was limited, resulting in increasing of biosurfactant production.

Thin Layer Chromatography (TLC) of Biosurfactant from *H. elongata* BK-AG18

The cell-free supernatant containing biosurfactant was precipitated using NaOH resulting crude biosurfactant. After that, the crude of biosurfactant was collected and extracted using chloroform/methanol (2:1). To analyze the purity of biosurfactants, thin layer chromatography (TLC) was carried out. The biosurfactants that have been eluted using chloroform/methanol/water (65:25:4) showed a single spot with R_f value about 0.7 under UV detection (Fig. 3). It appears that the extracted biosurfactant was relatively pure. This R_f value was similar to lipopeptide-type biosurfactant produced by Kocuria marina BS-15 [12]. The other halophilic bacteria, Halomonas sp. BS4, produced glycolipid biosurfactant with R_f value of 0.4, while the other glycolipid type known as rhamnolipid has an R_f value of about 0.7 [9]. Therefore, regarding polarity, the biosurfactant produced by H. elongata BK-AG18 is relatively close to lipopeptide and rhamnolipid.

Physicochemical Characteristic of Biosurfactant from *H. elongata* BK-AG18

Due to the presence of amphiphilic moieties, biosurfactants can reduce the surface tension between two molecules with different polarities such as air/water. In this study, the surface tension of air/water was measured with a Du Nouy tensiometer [21]. Fig. 4 showed the plot of surface tension versus biosurfactant concentration. The increasing of biosurfactant concentration was initially followed by the gradual decreasing of the surface tension of water, which was from 70.2 to 53.3 dyne/cm. The surface tension remained constant at the concentration greater than 275 mg/L

Fig 3. TLC of biosurfactant with R_f value of 0.7

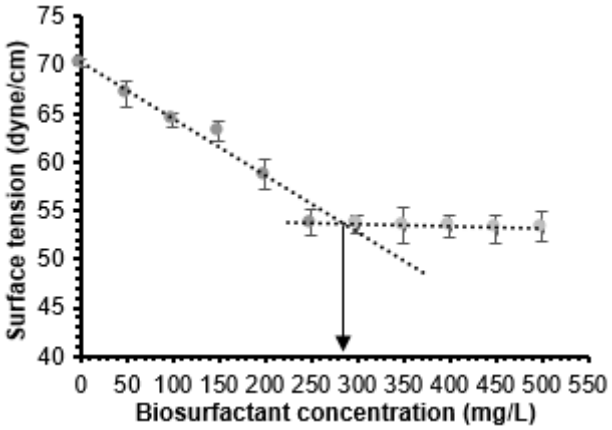

Fig 4. Surface tension measurement. Arrow line shows the CMC of biosurfactant

Fig 5. Emulsification activity (EI_{24}) of biosurfactant from *H. elongata* BK-AG18

indicating that the CMC value of the biosurfactant was about 275 mg/L. At CMC, the surface tension of water decreased about 16.9 dyne/cm. Hence it can be categorized as the potential surfactant because it can reduce the surface tension of water greater than 10 dyne/cm [22]. This CMC was similar to that glycolipid biosurfactant produced by Halomonas sp. MB-30 that was about 250 mg/L [8]. The similarity of CMC indicated the similarity of an amphipathic moiety in both biosurfactants.

The other characteristic of biosurfactants is the capability to emulsify the oil. Emulsification activity of biosurfactant can be determined by measuring the stabilization of emulsions formed within a period [6]. In this work, the emulsification activity of biosurfactant at CMC was studied against palm oil. The emulsion formed was allowed to stand for 24 h, and then the emulsification index (EI_{24}) was calculated by using the eq. 1 (Fig. 5). The calculation gave EI_{24} of the biosurfactants about 53.6%, which is relatively small for the emulsification activity. It is likely that the produced biosurfactant was predicted to have a relatively small molecular mass as stated by De et al. [23]. However, this result was higher compared to the value of EI_{24} of biosurfactants produced by the other halophilic bacteria such as Aerococcus sp., Kurthia sp., Coprococcus sp., Halococcus sp., and Clavibacter sp., which were about 17.22, 46.1, 22.21, 43.05, and 15.05%, respectively [12].

Partial Structural Characterization of Biosurfactant from *H. elongata* BK-AG18

The FTIR is an important analysis to identify the functional group of biosurfactant. The FTIR spectrum was depicted in Fig. 6 that showed a broad peak around 3450 cm^{-1} that indicates characteristic of –OH group. The peak around 2962 and 2362 cm^{-1} indicates the aliphatic chains (CH_3 and CH_2) stretching vibration. The peak at 1632 cm^{-1} confirms the presence of C=O stretching bond of unsaturated esters whereas at 1462 cm^{-1} is referred to the aliphatic chain of C-H group. The presence of a C-O-C stretch of the ester is found in the region around 1030 cm^{-1}. An absorption band around at 991 cm^{-1} is identified as the stretching mode of the $CH=CH_2$. This FT-IR spectrum was similar to the glycolipid type of biosurfactant obtained by Dhasayan et al. [8].

The further structural analysis was carried out by ^{1}H-NMR. The obtained ^{1}H-NMR spectrum of the biosurfactants showed some proton signals indicating the presence of fatty acid (lipid) and sugar moieties (Fig. 7). The chemical shift at 1.0-2.0 ppm was typically known as multiplet proton assigned to a fatty acid moiety,

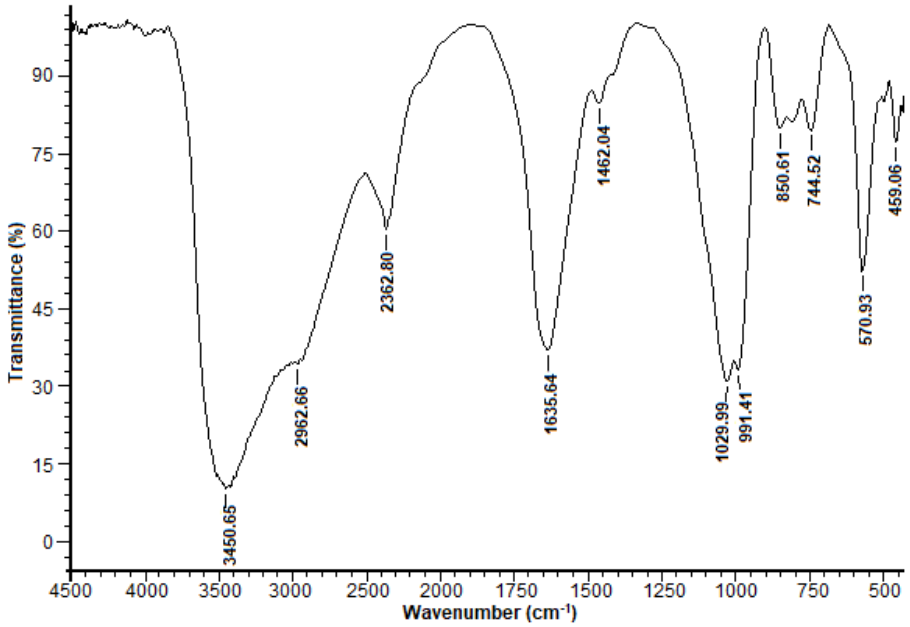

Fig 6. FTIR spectra of biosurfactant from *H. elongata* BK-AG18

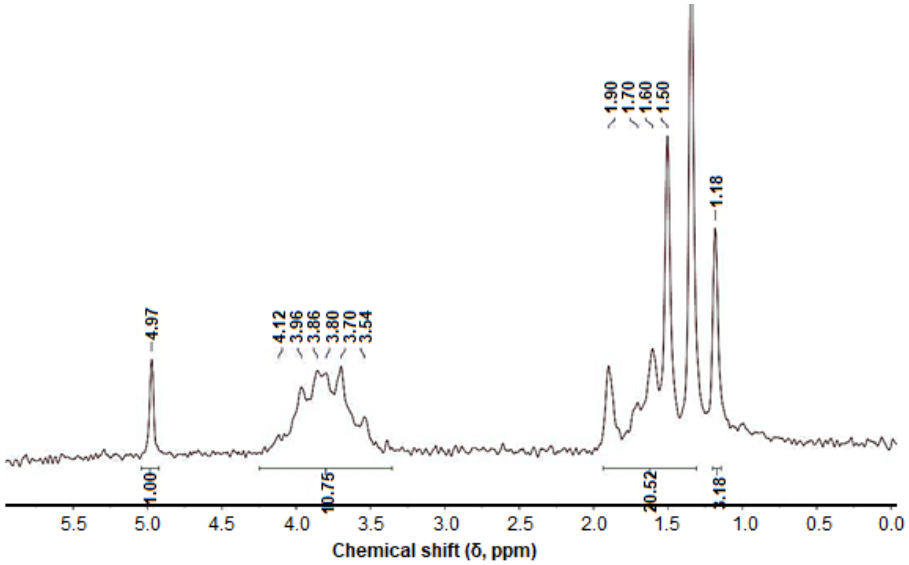

Fig 7. ^{1}H-NMR spectrum of *H. elongata* BK-AG18

while the chemical shifts at 3.5–4.2 ppm indicated the presence of glycosidic linkage of sugar. Moreover, at a chemical shift of 5.0 ppm, there was a singlet peak predicted as anomeric proton of the sugar (carbohydrate) moiety. Based on these results, biosurfactant from H. elongata BK-AG18 was predicted to have nonpolar part of fatty acid and polar part of sugar. This analysis supported the FTIR analysis above that the biosurfactants obtained in this study likely belonged to the glycolipid group of biosurfactant.

Antibacterial Activity of Biosurfactant from *H. elongata* BK-AG18

It has been reported that some biosurfactant also showed antibacterial activity, such as the one reported by Donio et al. that found biosurfactant produced by *Halomonas* sp. BS4 was able to inhibit the growth of some pathogenic bacteria [9]. Because of the similarity of the bacterial genus to the one used in our study, which is *Halomonas*, the potential of the biosurfactant produced by *H. elongata* BK-AG18 was also investigated. Two pathogenic bacteria were used as the target of the study, i.e., *E. coli* and *S. aureus*. As the preliminary test, the two bacteria were grown in a medium containing 1000 mg/L of biosurfactant and in a medium without the addition of biosurfactant. The antibacterial activity was evaluated by comparing the value of OD_{600} after the bacteria were grown

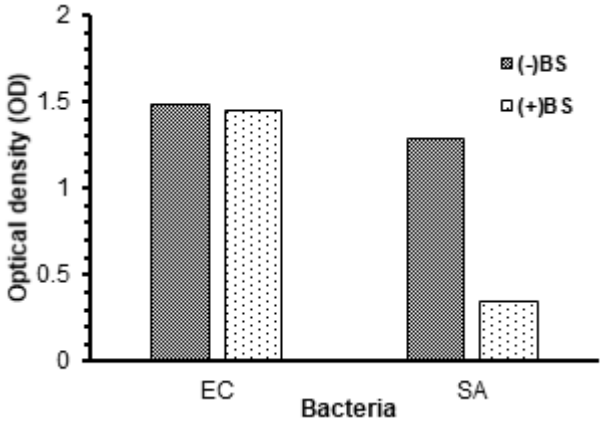

Fig 8. Growth profile of *E. coli* (EC) and *S. aureus* (SA) in the medium containing biosurfactants and without biosurfactants

for overnight. As shown in Fig. 8, the most significant decrease in OD_{600} value occurred in *S. aureus* growth. This indicated that biosurfactants produced by *H. elongata* BK-AG18 only effectively suppressed the growth of *S. aureus*. To precisely determine the minimum concentration of the biosurfactant that could inhibit *S. aureus*, the bacterium was grown in a serial medium containing different biosurfactant concentrations. By measuring OD_{600} in a different medium, it can be generated a plot of OD_{600} against biosurfactant concentrations, where the minimum inhibitory concentration (MIC) was determined at the

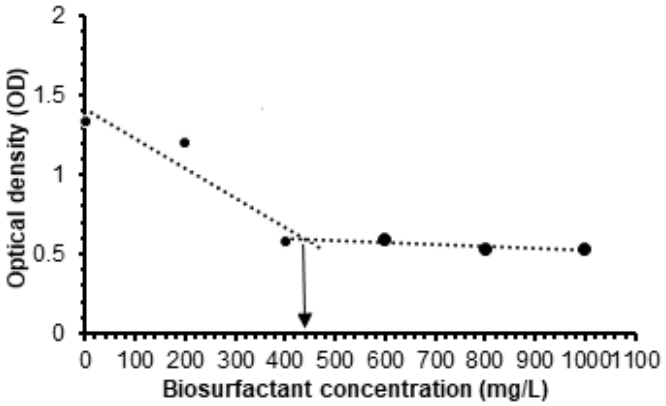

Fig 9. Growth profile of *S. aureus* in various biosurfactant concentration. Arrow line shows the minimum concentration of biosurfactants to inhibit the growth of *S. aureus*

breakpoint. Using this procedure, the MIC value for *S. aureus* was about 433 mg/L (Fig. 9). Some biosurfactants such as glycolipids and lipopeptides have been known to have antibacterial activity. Surfactin produced by *Bacillus licheniformis* strain M104 was able to inhibit the growth of *S. aureus* with MIC value of about 48 mg/L [24]. Antibacterial activity against *S. aureus* is also observed from glycolipid biosurfactants produced by *Staphylococcus saprophyticus* with MIC value about 12 mg/L [25]. Sophorolipid biosurfactants are also known to inhibit *S. aureus* with MIC value about 512 mg/L [26].

■ CONCLUSION

H. elongata BK-AG18 is the potential bacterium that able to convert glycerol into glycolipid type of biosurfactant. Biosurfactant produced exhibited a good emulsification activity and potential to be applied as an antibacterial agent.

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