Combination of Mollase and Glucose as Substrate for The Production of Biosurfactant by *Bacillus subtilis* BK7.1

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Abstract. Biosurfactant is a secondary metabolite which has properties and structures such as surfactants that are able to decrease surface tension water and cause microsolubilization or emulsification. Biosurfactants are active compounds that are produced at the microbial cell surface or excreted especially *Bacillus*. Previous research reported that *B. subtilis* BK7.1 had ability to form biosurfactant. In this study, *B. subtilis* BK7.1 produced biosurfactant using a combination of molasse and glucose as a carbon source. The purpose of study was characterized biosurfactant from *B. subtilis* BK7.1 by counting the emulsification index and the surface tension of supernatant, calculating (CMC) value, and examining stability of biosurfactant. *B. subtilis* BK7.1 could produce the biosurfactant from molasse and glucose with CMC value was about 4 g/L. Biosurfactant of *B. subtilis* BK7.1 could reduce the surface tension of medium from 54.68 to 49.2 mN/m, emulsify kerosene around 15.8%, had temperature stability in the range of 27ºC to 45ºC, and had stability at pH 6. This study showed that the use of molasses waste combined with glucose in biosurfactant production was very efficient and had potential for further applications.

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1 Introduction

Surfactants are amphipathic molecules because they consist of polar and non-polar groups so they have the ability to form emulsions and reduce surface tension [1]. Surfactants have been used extensively in industry as detergents and solvents and also have applications in the food, agricultural and pharmaceutical fields [2]. However, chemically synthesized surfactants are toxic to the environment, difficult to degrade, and strip away the skin's natural moisture and accelerate the aging process of human skin [3].

Biosurfactants are amphiphilic surface active compounds produced by microorganisms, including molds, yeasts, and bacteria [4]. Biosurfactants have hydrophobic and hydrophilic groups [5]. The unique characteristics of biosurfactants are lower toxicity, higher biodegradability, and environmental friendliness [6]. The advantages of these biosurfactants can replace the role of synthetic surfactants [7]. In addition, biosurfactants are also useful for antibacterial and anti-biofilm against bacteria such as *P. mirabilis*, *P. aeruginosa*, and *S. aureus* [8].

Biosurfactants produced by bacteria such as *Bacillus* are of greater interest because they are able to produce abundant biomass in a relatively short time, but the commercialization of biosurfactants is hindered because they require expensive substrates [9]. Efforts that have been made by the world to overcome these problems are exploration of cheap raw materials as substrates [10]. The use of glucose in biosurfactant production by *Bacillus* was very attractive because of the high yields, cheap, and renewable substrates. In addition, industrial waste such as molasses can also be a cheap alternative carbon source for biosurfactant production [11]. Many studies have reported the production of biosurfactants with a combination of substrates. However, research on the potential of *B. subtilis* BK7.1, isolated from Baluran National Park, in producing biosurfactants with a combination of two substrates has never been carried out. In this research, we focus on exploring the substrate combinations of molasses as a representative of industrial waste and glucose as a renewable substrate by *B. subtilis* BK7.1.

2 Material and Methods

2.1 Bacteria and isolate preparation

Bacillus subtilis BK7.1 was the culture collection of Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, Airlangga University, Indonesia. *Bacillus subtilis* BK7.1 was isolated from Baluran National Park soil samples [12]. The isolate was maintained aerobically on agar plates and was regularly transferred into fresh nutrient broth (NB) medium for shortterm storage. The isolate of *B. subtilis* BK7.1 was prepared by transferring a loop of cells from a slant culture to 50 mL of NB. The culture was incubated at room temperature in rotary shaker at 37°C, 120-150 rpm for 24 h.

2.2 Production of biosurfactant

The production medium was prepared by making minerals salt medium (MSM). The compositions of different media used in this study are (g/L): $(NH_4)_2SO_4$, 3; NaCl, 10; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.01; $MnSO_4.H_2O$, 0.001; H_3BO_3, 0.001; $ZnSO_4.7H_2O$, 0.001; $CuSO_4.5H_2O$, 0.001; $CoCl_2.6H_2O$, 0.005; dan $NaMoO_4.2H_2O$, 0.001. This solution was arrenged to pH 7.0 with adding NaOH 1 N or HCl 1 N. 470.4 ml of MSM was transferred to 1000 mL Erlenmeyer flasks and added by 1% glucose and 1% of molasses. Media were sterilized by autoclave at 121 °C for 15 min.

2.3 Determination of biomass concentration

Biomass was collected by culture centrifugation at 10.000 rpm for 10 min and the supernatant was separated from biomass. The biomass was dried at 80°C by oven for 24 h then determined the bacterial dry weight [13]. The formula for calculating dry cell weight is:

$$\text{DCW} = (\text{weight of dried cell biomass} - \text{weight of empty microtube}) / \text{volume of culture processed} \quad (1)$$

2.4 Measurement of surface tension

15 mL of supernatant was poured into a clean and sterile glass container and placed on sample table of tensiometer. The platinum ring was submerged right on the surface of supernatant and then pulled back slowly. The surface activities of the biosurfactants were determined by measuring the surface tension using Du Nouy's Tensiometer (Ogawa Seiki Co., Ltd) using the ring method at room temperature (25°C) [14].

2.5 Determination of emulsion formation capacity

Emulsifcation formation capacity was determined by calculating the emulsifcation index (E_{24}). Supernatant and kerosene (v/v) were placed into a measuring test tube and tighly closed. Those solution was vortexed vigorously for 1 min and then kept at room temperature for 24 h. The percentage (%) of the emulsion layer height (cm) divided by the total solution height was calculated as the emulsion index value (E_{24}) [14].

2.6 Crude biosurfactant extraction

A total of 1 liter of culture solution that has been incubated for 96 h at 37°C, then centrifuged at 6.000 rpm for 15 min to get the supernatant containing biosurfactant. After that, the crude biosurfactant extract was obtained by the method 60% ammonium sulfate deposition. The biosurfactant supernatant that had been obtained was put into a 1 L Beaker glass and immersed in an ice bath. Add ammonium sulfate slowly into the supernatant and while stirring with a magnetic stirrer until the ammonium sulfate level is 60% saturated. Stirring was carried out for 15 minutes, then centrifugation was carried out to obtain a precipitate of the biosurfactant crude extract biosurfactant [15].

2.7 Measurement of critical micelle concentration (CMC)

CMC have definition that minimum concentration of biosurfactant to initiate micelle formation and generally correlates with a constant surface tension value. CMC values are obtained in units of gr/L. Crude biosurfactant with concentration 1 g/L was prepared then to obtain a concentration of 2-7 g/L, dilution was carried out [16].

2.8 The stability of biosurfactant

Biosurfactant stability from *B. subtilis* BK7.1 was analyzed by determining the effect of pH, salinity, and temperature on biosurfactant activity. Determination of pH effect was done by making the crude biosurfactant solution at CMC point with various of pH value from 1 until 14. Determination of pH effect was done by making the crude biosurfactant solution at CMC point with various of salinity from 0% until 10%. To determine the temperature effect, the crude biosurfactant solution at CMC point were heated at 27°C until 80°C for 60 min. After

cooled to room temperature, surface tension was calculated by Du Nouy's Tensiometer. All experiments were the mean of triplicate analyses.

3 Result and Discussion

3.1 Culture conditions and biosurfactant production

Like bioethanol, biosurfactants could be produced sustainably through fermentation with the help of microbes such as *Bacillus* in this research using a combination of molasses and glucose [17]. The result from pH and biomass profiles MSM with 1% molasse and 1% glucose as carbon sources, have been showed in **Fig. 1**. *B. subtilis* BK7.1 was able to produce biosurfactant in an exponential phase with a maximum after an incubation time of 48 hours. The graph showed that *B. subtilis* BK7.1 used molasse and glucose as carbon sources to grow and produce the biosurfactants. The graph showed that the cultivation medium accomplished a decrease in pH. It was assumed that the bacteria could consume sugar in glucose or molasses during the first to fifth day of incubation. This was due to the formation of organic acids such as carboxylic acids which will produce H^+ ions when they dissociated with water [18].

Using glucose as a carbon source is favored by most heterotrophic bacteria. However, microorganisms had the ability to utilize a variety of other compounds as a carbon source for energy production [19]. Glucose was the best carbon source compared to carbohydrate and other hydrocarbon carbon sources which can reduce surface tension by 31.06 ± 0.54 mN/m [10].

The use of molasses combined with glucose in *B. subtilis* BK7.1 was due to the high cost of substrates in producing biosurfactants. Molasses used in making biosurfactants could reduce production costs by around 10-30% [20]. In addition, molasses was a low-cost and renewable substrate, a by-product of the cane sugar industry, rich in carbohydrates, vitamins and minerals making it very suitable for microbial consumption without the need for pre-treatment costs [21].

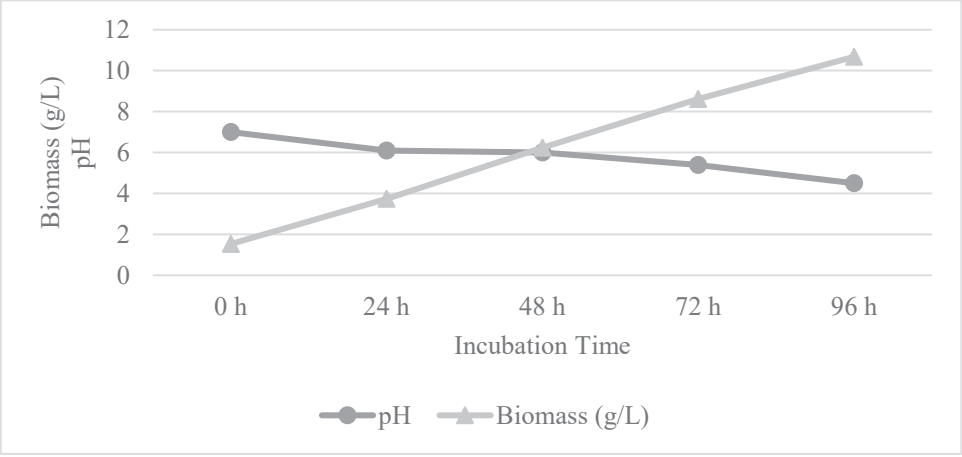

Fig. 1. The result of pH and biomass profiles from *Bacillus subtilis* BK7.1 in MSM with 1% molasse and 1% glucose as carbon sources

The result from surface activity and emulsification index profiles have been showed in **Fig. 2**. Biosynthesis of crude biosurfactant *B. subtilis* BK7.1, evaluated by measuring emulsification activity and surface tension. The results obtained were that there were both activities, both

surface tension and emulsification, from the first day of incubation until it reached an increase in the stationary phase. The graph also illustrated that the biosurfactant produced by the isolate was able to reduce the surface tension of the media from 54.68 mN/m to 49.2 mN/m. In addition, *B. subtilis* BK7.1 had an emulsification ability of 15.8%, but not too big value. This indicated that the biosurfactants produced by *B. subtilis* BK 7.1 have surface tension lowering and emulsifier properties. This also explained that there were relationships between substrate use, growth, and biosurfactant production.

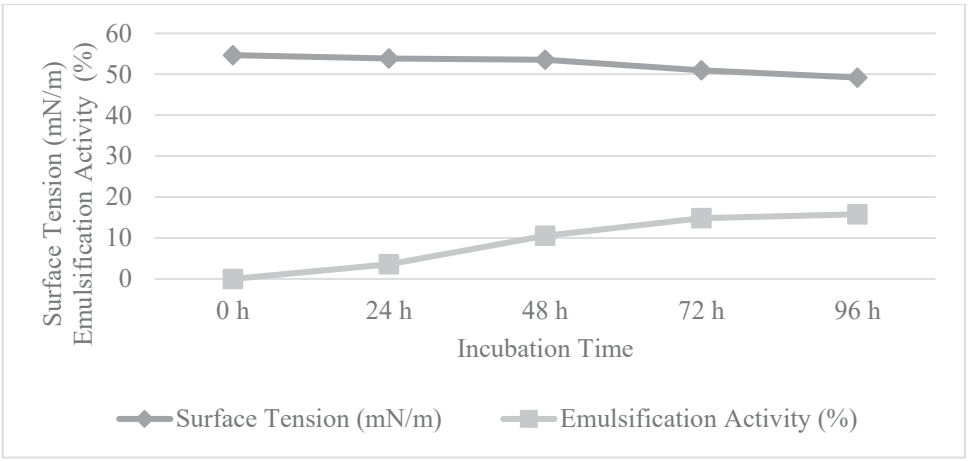

Fig. 2. The result of surface activity and emulsification index profiles from *Bacillus subtilis* BK7.1 in MSM with 1% molasse and 1% glucose as carbon sources

3.2 Effect of physicochemical factors on biosurfactant activity

Several environmental factors such as pH, temperature, and salinity can influence the efficiency and effectiveness of biosurfactants produced by microbes. The pH variation was carried out to determine the pH tolerance limit when applied in the environment. The results of characterization of the activity of biosurfactant products *B. subtilis* BK 7.1 was presented in **Fig. 3**. for pH. These results could be utilized in the naphthalene bioremediation process, in which pH in an alkaline state could increase solubility, due to ionic strength which can change the shape of the biosurfactant to become flat and vesicular so that it can increase the solubility of hydrocarbons due to the formation of micelles between ions and biosurfactant [22]. The decrease in surfactin micelle micropolarity could be caused by an increase in pH due to the dissolution of a large number of fluorescence probe molecules into the surfactin micelle core. Additionally, Ca^{2+} binding to the head group region of surfactin micelles causes an increase in micropolarity, thereby inhibiting the solubilization of the fluorescence probe into the micelle core [23].

The stability of crude biosurfactant from *B. subtilis* BK7.1 was determined to different salinity percentage with surface tension as response variable. Biosurfactant *B. subtilis* BK7.1 did not have halophilic characteristic that have been showed in **Fig. 4**. Salinity played an important role in the structure formation and solubility of biosurfactants. High salinity caused a reduction in the effectiveness of biosurfactants in forming emulsions and reduces interfacial tension [23]. In addition, the solubility of most surfactants in the aqueous phase is usually reduced at high brine salinity [24].

Temperature variations in the environmental also influence the activity of the crude biosurfactant *B. subtilis* BK7.1, which is visualized in **Fig. 5**. Biosurfactants produced by *Bacillus* to be more susceptible to changes in temperature affect the interactions between surfactin molecules, causing the formation of intra-hydrogen bonds within the surfactin molecules and inter-hydrogen bonds between them. One type of biosurfactant produced by these bacteria, surfactin, tends to form a flat structure and horse saddle conformation when temperature increases [23].

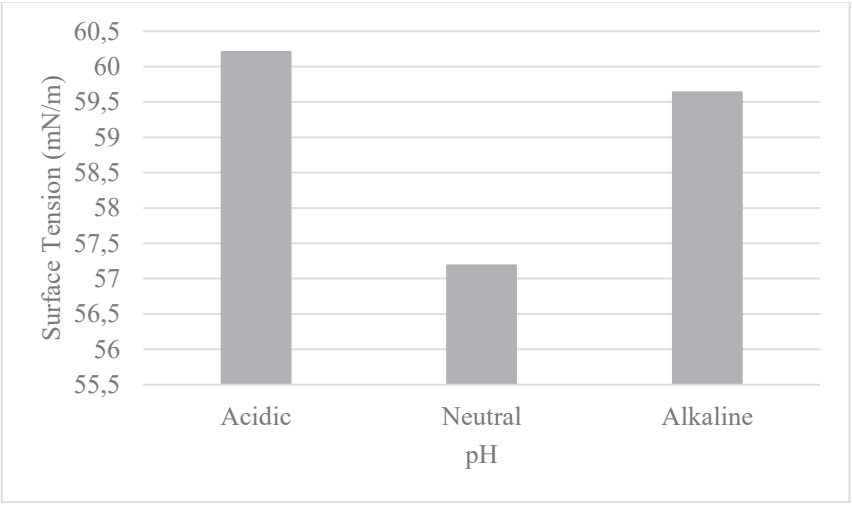

Fig. 3. The result of product activity from biosurfactant *Bacillus subtilis* BK7.1 against variations in pH

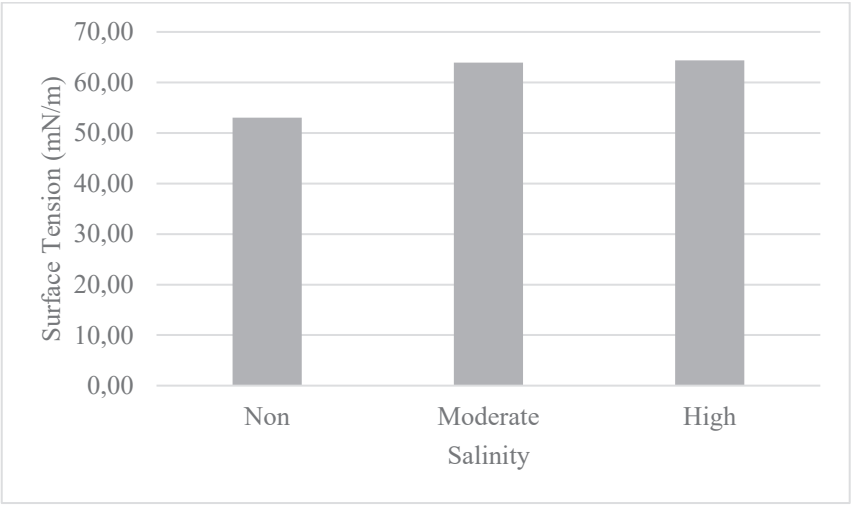

Fig. 4. The result of product activity from biosurfactant *Bacillus subtilis* BK7.1 againts variations salinity.

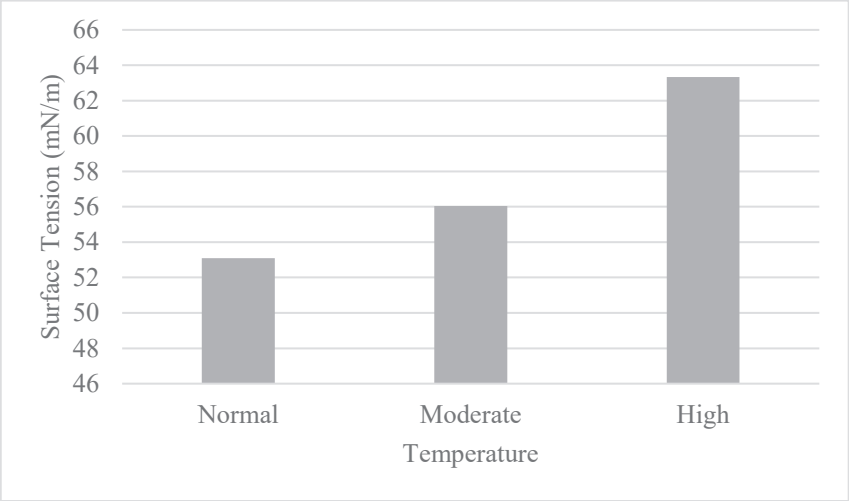

Fig. 5. The result of product activity from biosurfactant *Bacillus subtilis* BK7.1 against variations in temperature

4 Conclusion

This research showed the potential of isolate *Bacillus subtilis* BK7.1 in producing biosurfactant effectively with combination 1% molasse and 1% glucose as carbon sources. *B. subtilis* BK7.1 could produce the biosurfactant with CMC value was about 4 g/L. Biosurfactant of *B. subtilis* BK7.1 could reduce the surface tension of medium from 54.68 to 49.2 mN/m, emulsify kerosene around 15.8%, had temperature stability in the range of 27ºC to 45ºC, and had stability at pH 6. This study showed that the use of molasses waste combined with glucose in biosurfactant production was very efficient and had potential for further applications.

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