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Production of Biosurfactant Using *Bacillus subtilis* Natto Fermentation

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ABSTRACT

Biosurfactants are microbial amphiphiles produced as primary metabolites by varieties of microorganisms. They are preferred over chemically derived surfactants owing to their intrinsic properties, such as superior environmental compatibility, biodegradability, anti-inflammatory and antimicrobial activity, and higher tolerance towards extreme environmental conditions such as temperature, salinity, and pH levels. However, commercial production of biosurfactants is still lacking. The main reason for this is the low yields obtained from fermentation processes, which causes them to be unable to compete compared to chemical surfactants. The present study conducted a one-factor-at-a-time (OFAT) analysis on fermentation conditions to enhance biosurfactant yield from a probiotic strain, *Bacillus subtilis* Natto. The fermentation was conducted by varying parameters such as nitrogen source, vegetable oils, inoculum size, amino acids, and pH of the fermentation medium. Results showed a significant improvement of 45% in biosurfactant production from *B. subtilis* Natto when the initial pH of the fermentation medium was adjusted to pH 6.8, urea as the nitrogen source, inoculum size of 6% v/v and the addition of palm olein at a concentration of 2% v/v as a substrate in the fermentation medium.

Keywords: *B. subtilis* Natto, biosurfactant production, fermentation, OFAT analysis

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INTRODUCTION

The development of a new sustainable blueprint in microbial technologies to transform biomass into environmental-friendly carriers is needed for the sustainability of energy and the environment (Liu, 2020). Biosurfactants synthesized by microorganisms are amphipathic molecules consisting of hydrophilic heads and hydrophobic tails, normally hydrocarbon chains of lipids or fatty acids (Banat et al., 2014). They are categorized into three different types such as glycolipids (glucose and lipid), lipopeptides (protein and lipid), and phospholipids (phosphate and lipid) (Singh et al., 2018). These surface-active molecules can reduce surface or interfacial tension in liquids, exhibiting high emulsifying properties, and are stable at extreme pH, salinity, and temperature (Sharma et al., 2018). Compared to synthetic surfactants, they are less toxic, highly biodegradable, lower critical micellar concentration value, and are highly tolerant of extreme temperatures and pH (Varvaresou & Iakovou, 2015). Due to their intrinsic properties, they are particularly preferred in pharmaceutical, food, and cosmetic industries and environmental bioremediation (Bhattacharya et al., 2017; Felix et al., 2019).

A group of biosurfactants known as lipopeptides is commonly produced by *the Bacillus* genus due to their noteworthy efficiency and wide commercial applications (Hentati et al., 2019). There are three different families of lipopeptides: surfactins, iturins, and fengycins. These can be identified based on their multiple homologous and isoforms with different amino acid sequences and varied fatty acid chains (Ibrar & Zhang, 2020). In the biosynthesis of surfactin, the synthetase consists of four enzymatic subunits (SrfA, SrfB, SrfC, and SrfD). They are the catalysts in the nonribosomal mechanism, which incorporates specific amino acids into peptides and modules (Jahan et al., 2020). These bioactive lipopeptides can be applied in various biotechnological and biopharmaceutical fields owing to their ability to act as antibiotics, antiviral, antitumor agents, bioremediation and oil recovery agent for polluted crude-oil sites, disinfectant, and harmless to normal cells (PBMC and PC12) (Balan et al., 2017; Fanaei & Emtiazi, 2018; Yuliani et al., 2018).

Current fermentation processes to produce lipopeptides cannot fulfill industrial demand due to low production yield. Many efforts to improve lipopeptide production are reported, such as using other cheaper and more effective substrates from agro-industrial waste (bagasse), but the yield is still unsatisfactory (Das & Kumar, 2019; Liu et al., 2020). This issue motivated the application of vegetable oil derived from palm and coconut in biosurfactant production from *Bacillus subtilis* natto, as the exploration of these substrates is yet to be reported for this strain. These vegetable oils have been reported to increase biosurfactant yield for other strains, such as *Starmerella bombicola* and *Pseudomonas aeruginosa* (Hirata et al., 2021).

This study aims to conduct OFAT analysis of fermentation parameters in the small-scale production of biosurfactants from *B. subtilis* Natto culture. *B. subtilis* Natto is a food-grade

strain and has been traditionally used as a probiotic food supplement. It would be a novel finding to explore the ability of this strain not only as a probiotic but also as a biosurfactant producer. A recent study in 2019 reported on the use of this strain to produce surfactin, one type of biosurfactant. They reported a high yield of surfactin using enriched media (Landy medium) with the incorporation of attapulgite powder (Sun et al., 2019). This paper aims to assess the potential of vegetable oils, such as palm-based oils, which are widely available in Malaysia, and to investigate their effect on biosurfactant production from *B. subtilis* Natto. The production is expected to improve when the fermentation parameters from OFAT analysis are identified.

MATERIALS AND METHODS

Materials

Bacillus subtilis Natto spp. was procured in the form of food-grade powder from Isetan Japanese supermarket (Kuala Lumpur, Malaysia). Nutrient broth and agar powder were purchased from Sigma Aldrich (New Jersey). Meanwhile, to produce biosurfactants from *Bacillus subtilis* Natto, only analytical grades of chemicals and solutions were used. The compositions of growth media are 0.5% w/v sucrose (R&M Chemicals, U.K.), modified mineral salts medium comprised of 0.4% w/v mono-potassium hydrogen phosphate (Bio Basic, Canada), 1.4% w/v disodium hydrogen phosphate dodecahydrate, 0.02% w/v magnesium sulfate heptahydrate, 0.0002% w/v manganese sulfate monohydrate, 0.0001% w/v ferrous sulfate heptahydrate (R&M Chemicals, U.K), 2% w/v bacteriological peptone (Oxoid, U.K.) and 0.05% w/v yeast extract (Fischer BioReagents, U.S.A.). Different types of vegetable oils, a substrate for biosurfactant production used were palm kernel oil, palm-based olein (donated by Sime Darby Technology Centre, Malaysia) and coconut oil (purchased from Ayam Brand, Malaysia). Leucine and glutamic acid precursors were donated by Sime Darby Technology Centre, Malaysia.

Hydrochloric acid, sodium hydroxide (Sigma-Aldrich, New Jersey), and ethyl acetate (R&M Chemicals, U.K) were used for biosurfactant extraction. HPLC grade of methanol and acetic acid (R&M Chemicals, U.K) were used for biosurfactant quantification analysis.

Microbial Growth for Inoculum Preparation and Biosurfactant Production

B. subtilis Natto strain was stored at -80°C in 20% v/v glycerol stock solution after growing in a nutrient broth (NB) medium at a 1% w/v for 24 hours. For seed culture preparation, 1% v/v of stock solution was placed into NB medium in an Erlenmeyer flask (250 mL) with 100 mL of working solution. It was incubated at 37°C under orbital stirring at 150 rpm for 16 hours.

The strain was cultivated in a modified Cooper's basic mineral salts medium with the following composition: sucrose (20 g/L), yeast extract (0.5 g/L), $MgSO_4.7H_2O$ (0.8 mM),

$Na_2HPO_4.12H_2O$ (40 mM), KH_2PO_4 (30 mM), $MnSO_4.H_2O$ (10 µM) and $FeSO_4.7H_2O$ (4 µM) plus different nitrogen sources such as peptone, urea, ammonium chloride and sodium nitrate at variable concentrations to produce the biosurfactant (Kim et al., 1997). The initial pH of the medium was then adjusted to pH 6.8 before autoclaving at 121°C for 15 minutes. For small-scale operations, inoculum placed into the medium was 2% w/v in the Erlenmeyer flask with a working volume of 100 mL Tests were carried out on a very small scale as oxygen access to the medium was ensured. Incubation was carried out at conditions as shown in Table 1.

Table 1
Fermentation conditions of medium for the production of biosurfactant from B. subtilis *Natto as control*

Parameter	Description
Temperature	37°C
Duration	24 hours
Agitation speed	150 rpm
Working volume	100 mL
Nitrogen source	Peptone
Inoculum size	2% v/v
Concentration of vegetable oil	0% v/v
Initial pH	pH 6.8

Selection of Nitrogen Sources, Vegetable Oils, and Amino Acids

Nitrogen sources: peptone, NH_4Cl, urea, and $NaNO_3$ were screened for medium formulation. The addition of NH_4Cl, urea, and $NaNO_3$ into the fermentation medium at the concentration (g/L) of 2.67, 3.00, and 4.25, respectively, except peptone, which was added at 20 g/L (Cao et al., 2009). Nitrogen sources are autoclaved together with other nutrients in a fermentation medium at 121°C for 15 minutes. The high peptone concentration in the media formulation could increase biosurfactant production (Bertrand et al., 2018).

Meanwhile, vegetable oils such as palm oil, palm kernel oil, and coconut oil were tested at a concentration of 2.0% (w/v) as substrates for biosurfactant production. The best-performance vegetable oil was tested at different concentrations (4.0, 6.0, and 8.0 % v/v).

Next, amino acids, such as leucine and glutamic acid, were added to the medium at a concentration of 0.1 mM (Liu et al., 2012).

Effect of Culture Conditions on Biosurfactant Production

OFAT analysis on biosurfactant production in the culture medium was further investigated using different inoculum sizes (4.0, 6.0, and 8.0% v/v) and different initial pH of the fermentation medium (pH 5.5, 6.0, 6.5, and 7.5).

Extraction of Biosurfactant

After 24-hour fermentation, the culture broth was centrifuged using a centrifuge model 5810 R (Eppendorf, Hamburg) at 10000 x g for 10 min at 4°C. Cell-free supernatant was then acidified to pH 2.0 with 3.0 M HCl solution to precipitate biosurfactant and left overnight in a chiller at 4°C. Then, it was centrifuged at 10000 x g for 10 min at 4°C. The precipitate was freeze-dried using CoolSafe Freeze Dryer (Scanvac, Denmark), followed by liquid-liquid extraction. In this extraction, the precipitate was dissolved in 0.1M NaOH solution and the pH was adjusted to pH 8.0 using 1.0 M HCl. An equal volume of ethyl acetate was then added to the solution. The mixture was shaken at 210 rpm for 24 hours at 30°C. The organic phase was removed and evaporated in a rotary evaporator (Eyela, Japan). This liquid-liquid extraction was repeated three times to ensure the complete removal of biosurfactants (Chen & Juang, 2008).

Analytical Determinations

In order to obtain dry cell mass, 100 mL samples were centrifuged using a centrifuge model 5810 R (Eppendorf, Hamburg) at 10000 x g for 10 min at 4°C. After centrifugation at 10000 x g for 10 minutes, the pellet (biomass) was further subjected to rinsing with distilled water and then re-centrifuged at 3000 x g for 10 minutes. These rinsing and centrifugation steps are repeated three times. It was then dried in an oven at 100°C for 48 hours, followed by a desiccator filled with silica gel at room temperature. It was then reported using gravimetric analysis as cell dry weight. The obtained biosurfactant was also reported using gravimetric analysis. Biosurfactant yield was calculated as biosurfactant mass obtained in a given sample volume. In gravimetric analysis, the cell and biosurfactant mass were weighed using an analytical balance (Sartorius, Germany). Cell dry weight and biosurfactant yield were calculated based on the formula used by previous researchers displayed in Equations 1 and 2, respectively (Li & De Orduña, 2010; Santos et al., 2018).

$$\text{Cell dry weight (g/ L)} = \frac{Dry\ cell\ mass\ (g)}{Volume\ of\ sample\ taken\ (L)} \quad (1)$$

$$\text{Biosurfactant yield (mg/L)} = \frac{Biosurfactant\ mass\ (mg)}{Volume\ of\ sample\ taken\ (L)} \quad (2)$$

Characterization of Biosurfactant

After acidic precipitation and biosurfactant extraction, samples were analyzed for oil displacement assay and emulsification activity of biosurfactant against palm-based olein. For the oil displacement assay, 5 ml of distilled water was placed inside the petri dish. 100 µL of vegetable oil was spread onto the surface, and a biosurfactant solution of 10 µL was placed onto the oil surface. The diameter of the oil displaced was then measured (Morikawa et al., 1993). Next, emulsification activity was performed by adding 2.0 ml of

palm-based olein into 2.0 ml of biosurfactant solution, followed by vortexing at high speed for 2 minutes. The emulsification index, E24, was later calculated as a ratio of the height of the emulsion layer to the total height of the mixture and expressed as a percentage in Equation 3 (Cooper & Goldenberg, 1987)

$$E24\ (\%) = \frac{\text{height of emulsified layer}}{\text{height of liquid layer}} \times 100\% \qquad (3)$$

Statistical Analyses

All measurements of dry cell mass, biosurfactant mass, oil displacement assay and emulsification index were performed in triplicate. The triplicate refers to the repetition of the assay in one test. Means and standard deviation were calculated with Microsoft Office Excel 2016. SPSS analysis software was computed for ANOVA analysis to determine the significance of data with α set at 0.05.

RESULTS AND DISCUSSION

OFAT Analysis on Fermentation Conditions to Produce Biosurfactant

This study aimed to find suitable conditions based on significant operating parameters affecting biosurfactant production using OFAT analysis. These parameters are carbon source, nitrogen source, vegetable oils, inoculum size and pH of the culture.

Effect of Nitrogen Source. Four nitrogen sources tested were peptone, ammonium chloride, urea, and sodium nitrite. From Figure 1, peptone yielded the highest cell mass (1.48 ± 0.08 g/L) but the lowest biosurfactant yield relative to nitrogen content (4.03 ± 0.11 mg/g nitrogen content). In contrast, ammonium chloride and sodium nitrate resulted in a similar cell mass and biosurfactant yield relative to the nitrogen content of around 1 g/L and 16 mg/g nitrogen content, respectively. Meanwhile, a medium with urea as a nitrogen source for biosurfactant production from *B. subtilis* Natto contributed to the lowest cell mass (0.69 ± 0.02 g/L). However, its biosurfactant yield relative to nitrogen content (21.11 ± 1.50 mg/g nitrogen content) was the highest. It showed that urea was the most suitable nitrogen source for *B. subtilis* Natto to produce biosurfactants.

Urea will be selected as a nitrogen source based on the OFAT analysis. It was because the biosurfactant yield from urea was the highest, and urea is the cheapest alternative nitrogen source compared to peptone, ammonium chloride and sodium nitrate (Zhang et al., 2016). There was an indication that all inorganic nitrogen sources (ammonium chloride, sodium nitrate and urea) and organic nitrogen sources (peptone) with yeast extract can induce the production of surface-active compounds by microbes and regulation of biosurfactant synthesis (Eswari et al., 2016). According to Purwasena et al. (2020), nitrate

needs to be broken into ammonium to be absorbed by the cells, while ammonium is ready to be consumed by the cells, which suggests that sodium nitrate and ammonium chloride are used by *B.* natto for cell maintenance. Meanwhile, Ibrar and Zhang (2020) revealed the amino acid sequence in biosurfactant produced from *the Bacillus* genus, which hypothesized that urea helped in the biosurfactant production as urea is a carbamide with two amide group joined by a carbonyl functional group. So, selecting urea as a nitrogen source would be an economical approach for biosurfactant production from *B. subtilis* Natto.

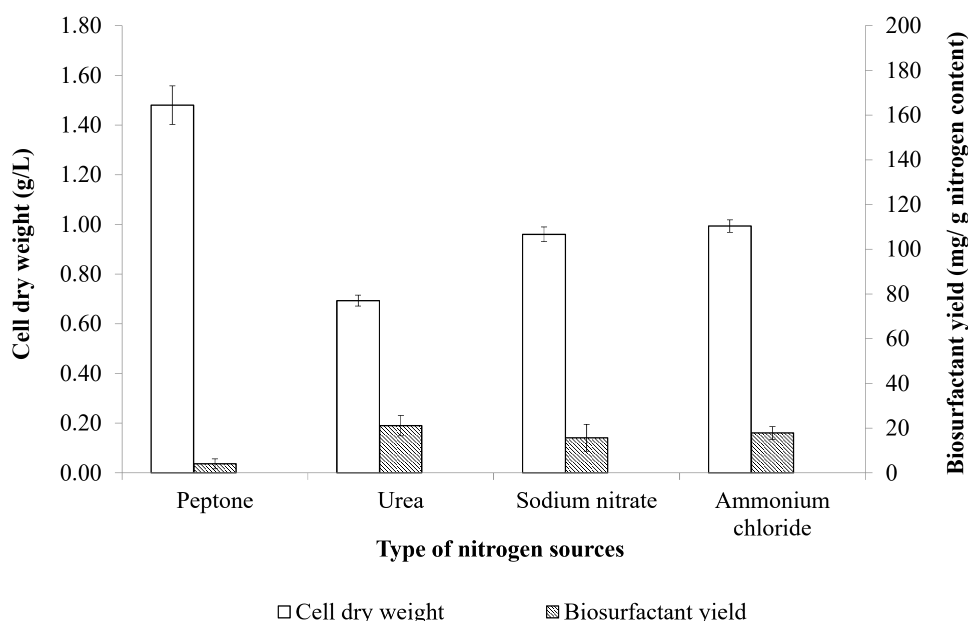

Figure 1. Effect of different nitrogen sources on biosurfactant production from *B. subtilis* Natto with sucrose as carbon source, inoculum size of 2% v/v and culture pH of 6.8

Effect of Vegetable Oils. The addition of vegetable oils (palm oil, palm kernel oil and coconut oil) at a concentration of 2% v/v into the medium increased the biosurfactant production from *B. subtilis* Natto by approximately two-fold. In Figure 2, the highest biosurfactant production (at 198.00 ± 9.90 mg/L) from *B. subtilis* Natto was obtained with palm kernel oil as substrate compared to palm oil and coconut oil, which yielded 167.33 ± 14.01 mg/L and 187.67 ± 10.66 mg/L, respectively. The medium supplemented with palm oil enabled cell mass production of 1.66 ± 0.01 g/L, while cell mass in culture with palm kernel oil and coconut oil was 1.53 ± 0.02 g/L and 1.53 ± 0.01 g/L of cell mass, respectively. Thus, palm kernel oil will be chosen as substrate added in the medium for biosurfactant production. This vegetable is also relatively cheap since it is abundantly available in Malaysia.

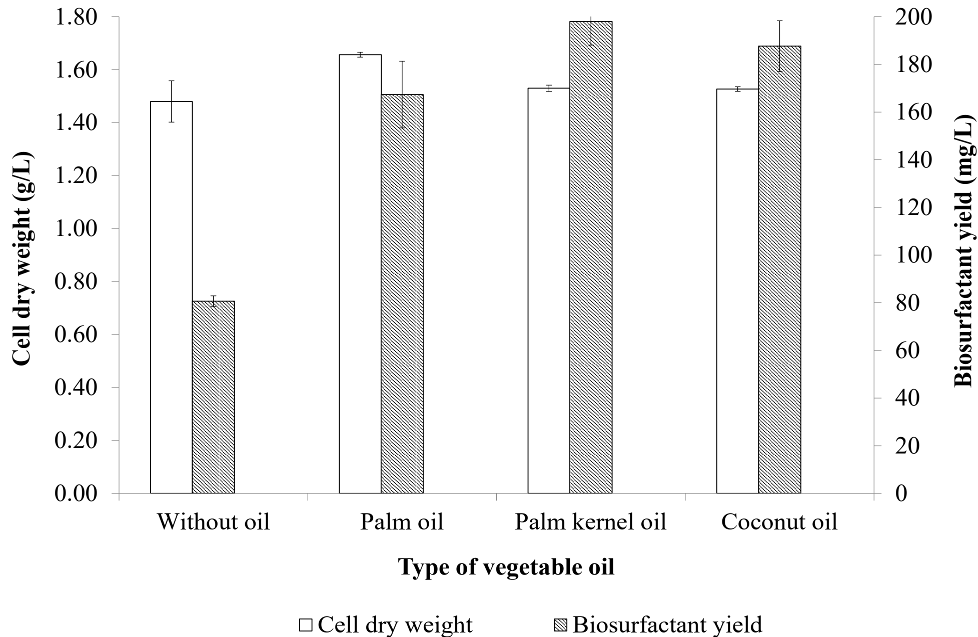

Figure 2. Effect of addition of different vegetable oil on biosurfactant production from *B. subtilis* Natto with sucrose as carbon source, peptone as nitrogen source, inoculum size of 2% v/v and culture pH of 6.8

Microbes fed on vegetable oils during the growth phase exhibited lipase activity to degrade vegetable oils into free fatty acid, mono and diacylglycerols. These compounds can reduce surface tension because they have surfactant properties (Ferraz et al., 2002). It also proved that biosurfactant produced by *B. subtilis* Natto was stimulated by adding the short-chain and long-chain fatty acids such as palmitic acid and lauric acid present in the fermentation medium. In agreement with past researchers who stated that nutrient uptake by the bacterial cell could be enhanced during the emulsification of these vegetable oils, leading to biosurfactant synthesis (Janek et al., 2010). These hydrophobic substrates also improved the yield of sophorolipids produced from *C. floricola* ZM1502 when incorporated into the medium (Konishi et al., 2018).

Effect of Different Concentrations of Vegetable Oils. In this part of the study, five different concentration of palm kernel oil was used to determine their effect on biosurfactant production from *B. subtilis* Natto. Figure 3 showed that increasing concentration from 0% to 2% v/v of palm kernel oil gave the highest cell and biosurfactant mass at 1.53 ± 0.02 g/L and 198.00 ± 9.90 mg/L, respectively. It showed that palm kernel oil was an inducer for biosurfactant production. Increasing palm kernel oil concentration from 2% v/v to 8% v/v resulted in a significant reduction in both cell mass and biosurfactant from 1.53 ± 0.02 g/L to 0.72 ± 0.03 g/L and from 198.00 ± 9.90 mg/L to 45.00 ± 2.83 mg/L, respectively. A

higher concentration of vegetable oil was found to inhibit the growth of *B. subtilis* Natto to produce biosurfactant because it caused a decrease in cell mass and biosurfactant. It suggested that an optimal concentration of vegetable oil is required as higher oils will most likely interfere with oxygen uptake by bacterial cells. The addition of vegetable oils at 2% v/v was chosen because it gave high biomass and biosurfactant yield and resulted in a low amount of residual oil at the end of fermentation. The lower the oil content on the fermentation harvest, the less likely it is to complicate subsequent downstream processes.

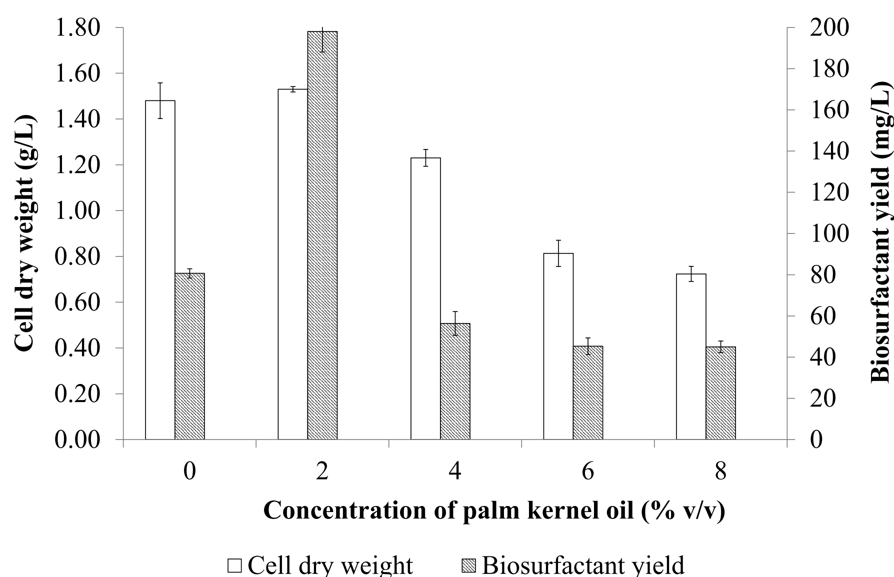

Figure 3. Effect of different concentrations of palm kernel oil on biosurfactant from *B. subtilis* Natto with sucrose as carbon source, peptone as nitrogen source, palm kernel oil as substrate, inoculum size of 2% v/v and culture pH of 6.8

By increasing palm kernel oil concentration above 2%, v/v inhibited the growth and production of biosurfactants from *B. subtilis* Natto. It indicated that substrates need to be supplied in optimal concentration for cell mass production and biosurfactant production. Khondee et al. (2015) and Thavasi et al. (2008) also reported using a low concentration of hydrophobic substrates (vegetable and crude oils) to enhance production from *Bacillus megaterium* and *Bacillus* sp. GY19. In this work, the amount of oil added to the reaction mixture had to be optimized to minimize the amount of oil left after fermentation for easier downstream processing.

Effect of Amino Acids. Two different amino acids (leucine and glutamic acid) were added into the medium to determine their effect on biosurfactant production from *B. subtilis* Natto. From Figure 4, the Addition of leucine to the culture medium caused a lower cell mass production (0.77 ± 0.01 g/L), but a slight increase of biosurfactant mass at 84.00 ± 4.55 mg/L was obtained. For a medium with glutamic acid, lower cell mass (1.14 ± 0.07 g/L) and decreased biosurfactant mass (76.33 ± 6.02 mg/L) were obtained. Although leucine improved the biosurfactant production (84.00 mg/L) compared to the medium without adding amino acids (80.67 ± 4.99 mg/L), the enhancement effect was insignificant. In other words, adding amino acid in the medium did not significantly enhance *B. subtilis* Natto to produce biosurfactants and help its growth. So, both amino acids would not be added to the medium for biosurfactant production from *B. subtilis* Natto.

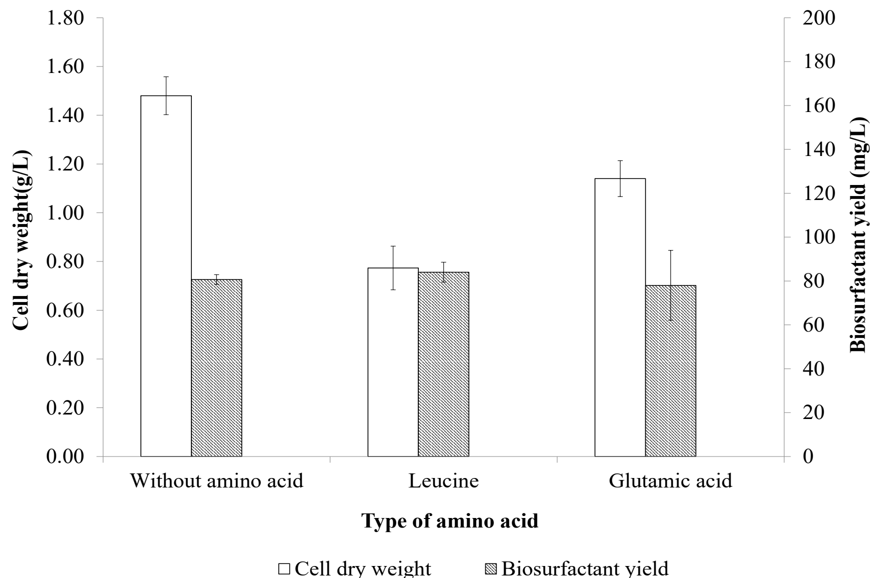

Figure 4. Effect of addition of leucine and glutamic acid on biosurfactant production from *B. subtilis* Natto with sucrose as carbon source, peptone as nitrogen source, inoculum size of 2% v/v and culture pH of 6.8

Another researcher reported that adding different amino acids did not improve surfactin production from *Bacillus subtilis* CS5 (Abdel-Mawgoud et al., 2008). However, there were other findings suggested that amino acids were suitable substrates for biosurfactant production by *S. ruminantium* and *Pseudomonas fluorescens,* which indicated that these two strains were able to yield more biosurfactant when grown in a medium supplied with amino acids (Saimmai et al., 2013; Biniarz et al., 2018). Therefore, from this finding, *B. subtilis* Natto did not require additional amino acid in the fermentation medium to produce

biosurfactant even though the biosurfactant produced by *B. subtilis* strains contains amino acids moiety consisting of leucine, valine, glutamic acid and aspartic acid (Pecci et al., 2010).

Effect of Inoculum Size. In Figure 5, *B. subtilis* Natto, with an inoculum size of 6% v/v, yielded the highest biosurfactant mass of 105.67 ± 11.15 mg/L and cell mass of 1.28 ± 0.07 g/L. Although an inoculum size of 2% v/v gave the highest cell mass of 1.48 ± 0.08 g/L, it yielded the lowest biosurfactant mass of 80.67 ± 2.25 mg/L. Figure 5 shows a decreasing trend of cell mass when inoculum size is increased from 2% to 4% v/v. When the inoculum size was further increased from 4 to 6% v/v, a gradual increase in cell mass from 1.15 ± 0.03 g/L to 1.28 ± 0.07 g/L was recorded. Nevertheless, when the inoculum was raised higher to 8% v/v, it caused a reduction in cell mass production at 1.19 ± 0.06 g/L. As for the yield of biosurfactant, increasing inoculum size from 2% to 6% v/v led to the increment in yield from 80.67 ± 2.25 mg/L to 105.67 ± 11.15 mg/L. Nevertheless, it dropped to 96.00 ± 6.98 mg/L in a higher % of inoculum size (8% v/v) culture. From this finding, 6% v/v is the most suitable inoculum size for use in the medium for biosurfactant production from *B. subtilis* Natto culture.

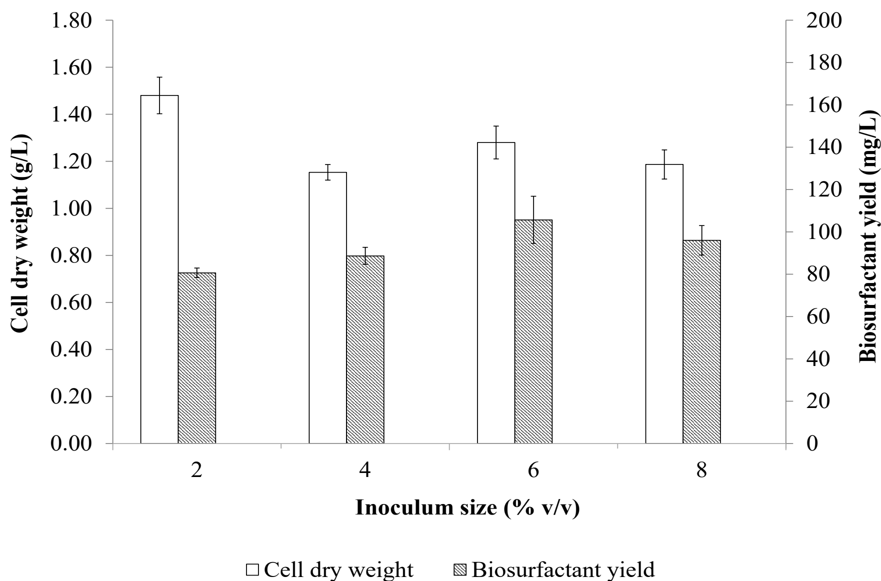

Figure 5. Effect of different inoculum sizes on biosurfactant production from *B. subtilis* Natto with sucrose as carbon source, peptone as nitrogen source and culture pH of 6.8

The maximum biosurfactant mass was recorded when *B. subtilis* Natto was inoculated into the medium at 6% v/v because inoculum size above 6% v/v decreased the mass of

biosurfactant produced. It showed the limitation of increasing inoculum size in improving microorganisms' growth and growth-related activities. When a lower inoculum size was used, the number of cells present in the production medium was low, which required a longer time to reach the exponential phase to utilize the substrate in the formation of the desired product (Nalini & Parthasarathi, 2018). So, selecting a suitable inoculum size in the production medium was important to maintain the balance between inoculum size and the media volume. As reported by previous work, increasing inoculum size would decrease microbial activity due to the limited availability of nutrients in the medium used by microorganisms (Korai et al., 2014).

Effect of Initial pH of Fermentation Medium. Five initial pH of fermentation medium (pH 5.5, 6.0, 6.5, 6.8 and 7.5) were tested for their effects on biosurfactant production. From Figure 6, as the initial pH of the fermentation medium increased from pH 5.5 to pH 6.8, cell mass increased from 1.38 ± 0.06 g/L to 1.48 ± 0.06 g/L while biosurfactant mass increased from 32.00 ± 2.16 mg/L to 80.67 ± 4.99 mg/L. When *B. subtilis* Natto was grown in a fermentation medium with an initial pH of 7.5, cell mass remained constant at 1.48 ± 0.02 g/L. However, biosurfactant production decreased to 60.33 ± 0.47 mg/L compared to the initial pH of 6.8 (80.67 ± 4.99 mg/L). So, a fermentation medium with an initial pH of 6.8 was selected for *B. subtilis* Natto in biosurfactant production. This initial pH of 6.8 provided a suitable environment for *B. subtilis* Natto to produce biosurfactant, as proven by its highest cell and biosurfactant mass.

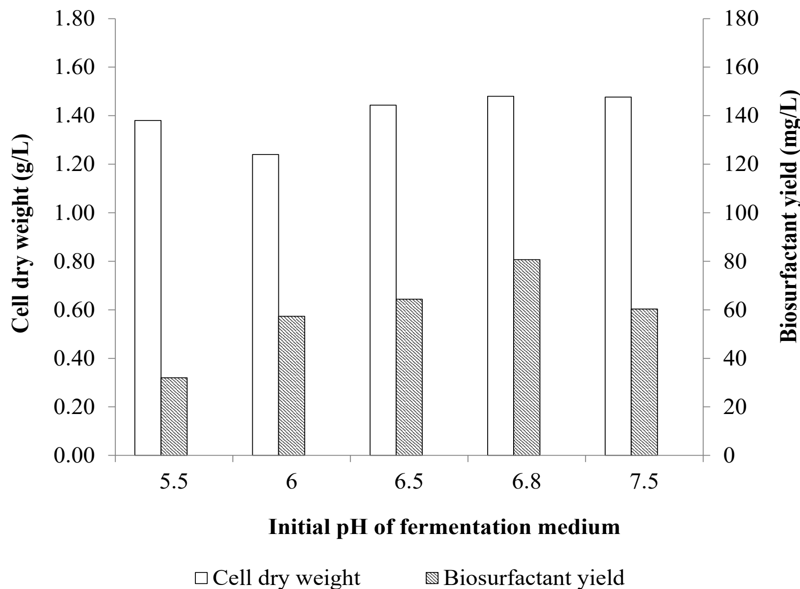

Figure 6. Effect of different initial pH of fermentation medium on production from *B. subtilis* Natto with sucrose as carbon source, peptone as nitrogen source, inoculum size of 2% v/v and culture pH of 6.8

There was a drastic decline in biosurfactant production at lower pH of 5.5 compared to higher pH of 7.5, which illustrated that acidic and alkaline medium provided an unsuitable environment for *B. subtilis* Natto to produce biosurfactants. However, biosurfactant mass was the highest at pH 6.8, indicating that a medium of nearly neutral pH was preferred for biosurfactant production from *B. subtilis* Natto. This result was in agreement with Kiran et al. (2010) and Vigneshwaran et al. (2018), who reported medium of neutral pH was suitable for biosurfactant production from *Brevibacillus* sp. and *Brevibacterium aureum*. So, the pH of the fermentation medium was important for bacterial growth because it can affect the absorption of nutrients, reproduction and activity of the enzyme of microorganisms (Zhang et al., 2015).

Fermentation Conditions from OFAT Analysis. Using ANOVA Analysis Tools, all the parameters were significant except for adding amino acid into the fermentation medium. It was because the p-value calculated for Figure 4 was 0.40, which was greater than the value of α set at 0.05. For the nitrogen source, urea was chosen over peptone and sodium nitrate for economic reasons, and the p-value shows that the biosurfactant yield was significant. Urea is the cheapest nitrogen source used for fermentation. When *B. subtilis* Natto was grown in the fermentation medium with the combination of the selected parameters, which are urea as nitrogen source, initial pH of 6.8 in the fermentation medium, inoculum size of 6% v/v, and addition of palm kernel oil at a concentration of 2% v/v, the yield for biomass and biosurfactant obtained were 1.56 ± 0.02 g/L and 362.33 ± 19.48 mg/L. The biosurfactant yield improved by 45.00% from 80.67 ± 4.99 mg/L to 362.33 ± 19.48 mg/L.

In Table 2, de Sousa et al. (2014) and Youssef et al. (2013) also reported low biosurfactant yield of 158.14 mg/L and 28.00 mg/L from *Bacillus subtilis* ATCC 6633 and *Bacillus subtilis* subsp. *subtilis spizizenii* NRRL B-23049, respectively. However, one researcher reported a higher biosurfactant yield of 657.23 mg/L from *Bacillus subtilis* subsp. *natto* NT-6 (Sun et al. , 2019). There was a disparity in the yield because the strain was grown in a Landy medium at 28°C for 36 hours. Another researcher reported a higher biosurfactant yield of 657 mg/L when they incorporated brewery waste into a nutrient salt medium (Moshtagh et al., 2018). So, this research provided a new insight that vegetable oil such as palm kernel oil which is abundant in Malaysia, exhibits the ability as a substrate for biosurfactant production from *B. subtilis* Natto. Food-grade *B. subtilis* Natto can act as a probiotic strain and biosurfactant producer.

Table 2

Comparison of biosurfactant yield from other researchers

Types of strain	Biosurfactant yield (mg/L)	Reference
Bacillus subtilis ATCC 6633	158.14	de Sousa et al. (2014)
Bacillus licheniformis RS-1	20	Youssef et al. (2013)
Bacillus subtilis subsp. *subtilis spizizenii* NRRL B-23049	28	
Bacillus subtilis subsp. *natto* NT-6	657.23	Sun et al. (2019)
Bacillus subtilis N3-1P	657	Moshtagh et al. (2018)

Characterization of Biosurfactant

The product obtained from *B. subtilis* Natto fermentation was subjected to qualitative characterization to confirm that it is a biosurfactant. Two of the characteristics of biosurfactants are oil displacement and emulsification ability. Figure 7 showed the product recovered from *B. subtilis* Natto was able to displace palm oil, with a clear zone measured 5.50 ± 0.08 cm. Meanwhile, Figure 8 shows the formation of an emulsion layer when *B. subtilis* Natto product was added to the water-oil mixture after vortexing. The emulsification index was calculated at 45.67 ± 2.49 %.

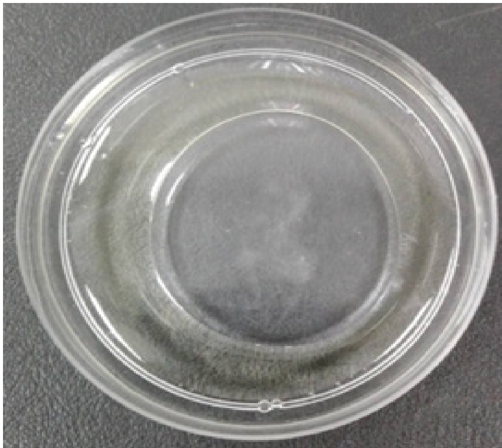

Figure 7. Oil displacement assay for biosurfactant produced from *B. subtilis* Natto

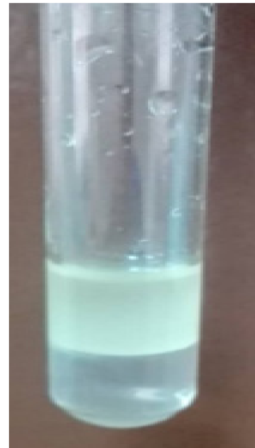

Figure 8. Emulsification assay for biosurfactant produced from *B. subtilis* Natto

The clear zone and emulsion formation indicated biosurfactant-producing capability by *B. subtilis* Natto. Besides, many researchers have used the oil displacement method to determine biosurfactant production efficiency because it depends on the decrease in water-oil interfacial tension regardless of biosurfactant structure (Parthipan et al., 2017). In this field, Anjum et al. (2016) reported the highest emulsification index for biosurfactant produced from *Bacillus sp.* MTCC 5877 was 76 ± 0.57%, while E24 of biosurfactant from *Bacillus cereus* and *Bacillus subtilis* were reported by Jaysree et al. (2011) with values ranging between 15 and 30% for diesel and engine oil, respectively Emulsification index obtained from this research was 45.67 ± 2.49%. It also suggested that biosurfactants produced exhibited a low ability to emulsify vegetable oil which can be used as an antimicrobial agent in nanoemulsion against food-borne pathogens (Joe et al., 2012).

CONCLUSION

OFAT analysis of biosurfactant production from *B. subtilis* Natto showed an improvement of 45% in biosurfactant yield when *B. subtilis* Natto was grown in fermentation medium using urea as nitrogen source, initial pH 6.8 of fermentation medium, with inoculum size of 6% v/v and addition of palm kernel oil at a concentration of 2% v/v into the fermentation medium. From the endpoint of fermentation results, the important parameters in enhancing the production are types of nitrogen source, inoculum size, types and concentrations of vegetable oils, and initial pH of the fermentation medium. Using ANOVA analysis tools, the most significant parameters to enhance production are vegetable oils. *B. subtilis* Natto can act as a probiotic strain and biosurfactant producer.

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