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Research article

Kinetic study and modeling of biosurfactant production using *Bacillus* sp.

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ABSTRACT

Background: Surfactants are one of the most important raw materials used in various industrial fields as emulsifiers, corrosion inhibitors, foaming agents, detergent products, and so on. However, commercial surfactant production is costly, and its demand is steadily increasing. This study aimed to evaluate the performance of typical strains of *Bacillus* sp. to produce biosurfactants through fermentation. It also included the investigation of the effect of initial glucose concentration and the carbon to nitrogen ratio.
Results: The biosurfactant yield was in the range of 1–2.46 g/L at initial glucose concentrations of 10–70 g/L. The optimum fermentation condition was achieved at a carbon to nitrogen ratio of 12.4, with a decrease in surface tension of up to 27 mN/m.
Conclusions: For further development and industrial applications, the modified Gompertz equation is proposed to predict the cell mass and biosurfactant production as a goodness of fit was obtained with this model. The modified Gompertz equation was also extended to enable the excellent prediction of the surface tension.

1. Introduction

A surfactant is a surface-active compound consisting of hydrophobic and hydrophilic components and is capable of merging two immiscible compounds such as water and oil through surface tension reduction [1,2]. It is one of the most important materials used in various industries, e.g., emulsifiers; corrosion inhibition, foaming, detergency, and hair conditioning industry; and in the enhancement of oil recovery, lubricants, and crude oil drilling [1,3]. The abundant demand of surfactant is fulfilled by a chemical compound derived from petroleum; however, the substance may cause toxicity problems to the environment, and it is non-biodegradable [3].

Biosurfactant as a natural surfactant produced by microorganisms from various substrates has attracted much more attention from industry [4]. This is due to its higher biodegradability, lower toxicity, environmental friendliness, and effectiveness under extreme conditions of salinity, pH, and temperature [5,6]. One of the most effective and powerful biosurfactants is surfactin, a lipopeptide type of biosurfactant produced by *Bacillus* sp. [5,7]. *Bacillus* sp. such as *Bacillus subtilis*, *Bacillus mojavensis*, *Bacillus pumilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* can synthesize surfactin [4,8,9,10]. Some strains of *Bacillus* sp. could decrease the surface tension from 72 to 27 mN/m during fermentation [3,5,7].

For the production of surfactin, various carbon sources such as glucose, sucrose, galactose, maltose, and mannitol have been utilized [11,12]. Sucrose was an optimal substrate to enable *Bacillus* sp. to produce surfactin [13]; this type of microorganism could also withstand high glucose concentrations [4]. Moreover, utilization of high initial sugar concentration is desirable to minimize the economic cost and prevent osmosensitive impurities in the fermentation broth [14,15]. Nitrogen is reasonably considered to be a nutrient source for the production of surfactin [4,13]. The effect of carbon to nitrogen (C/N) ratio is one of the most important parameters in biological systems and has been extensively studied in many fermentation processes [13,16,17].

Kinetic models are valuable tools to sustain further development of the process or industrial application [18]. It is because kinetic models can predict the profile of important products. The Monod kinetic model is usually used to describe the growth of yeast and substrate profiles [16,19]; however, in some processes, the model fails with regard to the prediction of products [20]. The modified Gompertz equation has been successfully applied to predict the production of ethanol during fermentation [21].

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The emphasis of this contribution is to study the effect of the initial glucose concentration on *Bacillus* sp. isolated from local environments, namely *Bacillus* sp. BMN 14 and BMN 27. In addition, the effect of C/N ratio was investigated. For further process development and large-scale applications, the modified Gompertz kinetic model was applied for the prediction of biosurfactant and microbe profiles. The model was also, for the first time, extended to enable the description of surface tension profiles in the fermentation broth. The prediction of the profiles of the cell and its products by kinetic models is useful as the basis for the calculation and evaluation of economic analysis during industrial applications [22].

2. Materials and methods

2.1. Microorganisms and inoculum media

Bacillus sp. BMN 14 and BMN 27 were isolated from soil contaminated with palm oil from a local area. The isolation process was based upon a method by Morikawa et al. [23]. Half gram of soil obtained from the land contaminated with crude palm oil (CPO) at the CPO industry in Banten, West Java, was poured in a small tube containing 9.9 mL sterilized water for 25 min. The solution was further diluted to 10^{-8} degree and incubated in agar media for 7 d at 37°C. The agar media contained 0.5% leavened extract, 0.5% NaCl, and 1% bacto-tryptone. The isolated bacteria were then grown in blood agar modified with the addition of 40 μL chicken blood in 10 mL media. The media contained 0.5% leavened extract, 0.5% NaCl, and 2% bacto agar. The growth and capability of the bacteria for producing biosurfactant was compared with the commercial bacteria *B. pumilus* JCM 2508 from Japan Collection of Microorganisms (JCM). Two strains of microbes, namely *Bacillus* sp. BMN 14 and BMN 27, were then selected depending on their ability to form an emulsifying zone around the colony and their capability to grow in comparison with those for *B. pumilus* JCM 2508. The microbes were later incubated in an incubator (MCO 175, Osaka, Japan) on agar slants for 24 h at 37°C [24]. A loop full of cells from the agar slant was then transferred to a 250-mL flask containing 50 mL sterilized propagation medium (PM) to be propagated in a water bath shaker (WNE 14, Memmert, Germany) at 140 rpm and 37°C for 24 h. The composition of the PM was as follows: minerals of 0.05 M NH_4NO_3, 0.03 M KH_2PO_4, 0.04 M Na_2HPO_4, 8×10^{-4} M $MgSO_4$, 7×10^{-6} M $CaCl_2$, and 4×10^{-6} M $FeSO_4$; 4×10^{-6} M Na_2EDTA; and glucose [1%, 3%, 5%, or 7% (w/v)].

2.2. Fermentation process

The fermentation processes were conducted in 250 mL Erlenmeyer flasks, with a working volume of 50 mL. The flasks were placed in a water bath shaker (WNE 14, Memmert, Germany) at 37°C and 140 rpm. The fermentation medium consisted of 10% PM and 90% substrate medium (SM). The SM used had the same mineral composition as the PM, but did not contain any cell. To observe the performance of strains BMN 14 and BMN 27, 4% (w/v) glucose concentration was used in the PM and SM. To determine the effect of the initial glucose concentration, 1%, 3%, 5%, and 7% (w/v) glucose was used for both PM and SM. To determine the effect of NH_4NO_3 concentration, 5% (w/v) glucose was used with NH_4NO_3 concentrations of 0.04, 0.05, and 0.06 M. These concentrations led to C/N ratios of 17.51, 12.36, and 10.56, respectively.

2.3. Samples analysis

Samples were routinely taken and centrifuged (Eppendorf 5427R, Hamburg, Germany) at 13,000 rpm for 10 min to separate the cell mass. The cell mass concentration was then quantified using the dry weight method. Prior to drying at 90°C for 24 h, the cells were washed with purified water and re-centrifuged. HCl was added to the fermentation broth to obtain pH 2. The acid precipitate was extracted with methanol [25,26] and then centrifuged at 3000 rpm for 15 min. The solution was filtered using a 0.2-μm Millipore membrane. The biosurfactant content in the filtrate was further analyzed using high-performance liquid chromatography (Model Shimadzu HPLC 10A VP, Shimadzu, Japan). The temperature of the column was maintained at 40°C, and acetonitrile solution with 1% acetic acid was used as the mobile phase at a flow rate of 1.5 mL/min. The glucose concentration was analyzed using a biochemistry analyzer (YSI 2700, Yellow Springs, OH, USA). The surface tension was evaluated using a tensiometer (Model 70545, CSC Scientific Co. Inc., Fairfax, VA, USA).

2.4. Critical micelle concentration

Critical micelle concentration (CMC) is the concentration of the surfactant at which no further decrease in surface tension is detected [27]. This parameter is important to examine the efficiency of the biosurfactant [8], and the minimum value of surface tension merely shows the effectiveness [3]. In this study, the CMC was determined by a graphical technique, i.e., by plotting the surface tensions (Y-axis) against surfactin concentration (X-axis). The CMC point was further evaluated by vertically projecting a line from the graph to the X-axis. The CMC point was obtained from the intersection of two straight lines. The first straight line was formed using a linear regression based on data at high surface tension, while other line was formed using a linear regression method based on data at low surface tension.

3. Results and discussion

3.1. Performance of Bacillus sp. BMN 14 and BMN 27

Fig. 1 presents the kinetic profiles of cell mass, biosurfactant, and surface tension of glucose fermentation by *Bacillus* sp. BMN 14 and BMN 27. The type of biosurfactant produced by the strains BMN 14 and BMN 27 is surfactin (cyclic lipopeptide). As shown in Fig. 1, the strain growth on glucose increased during the early process of fermentation (first 18 h), followed by biosurfactant production, continuing to increase until the end of the process. The growth of both strains was inhibited after 18 h of fermentation; it is probably due to increase in biosurfactant production. The inhibition of cell mass growth at high product concentration is a common phenomenon in fermentation processes [28]. A substantial production of the biosurfactant was obtained with *Bacillus* sp. BMN 14 at 24 h in comparison to that with BMN 27. This implies that the biosurfactant

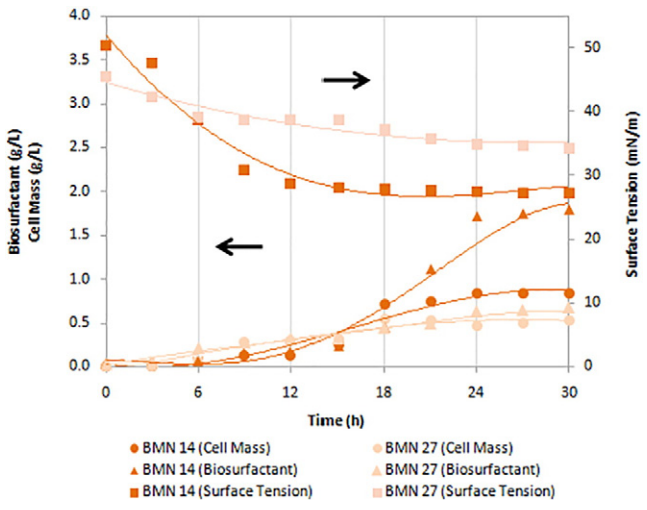

Fig. 1. Kinetic profiles of cell mass, biosurfactant, and surface tension for glucose fermentation using *Bacillus* sp. BMN 14 and BMN 27.

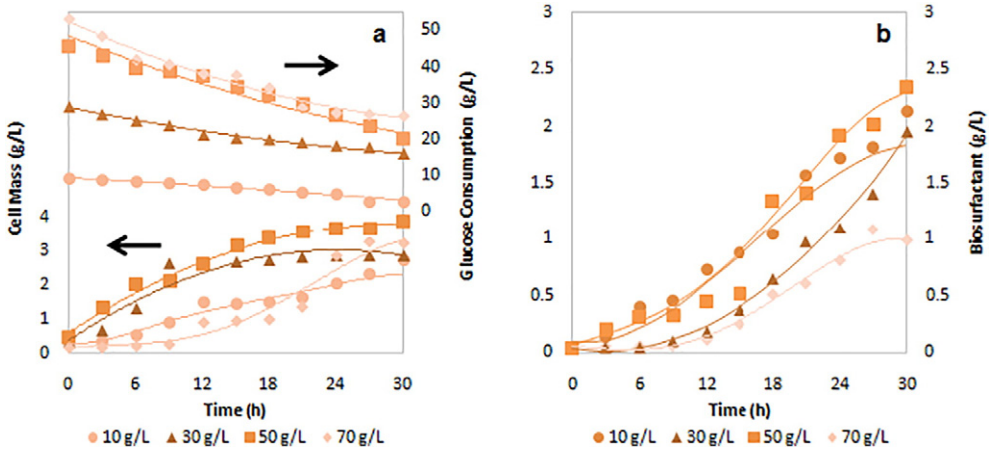

Fig. 2. Profiles of (a) cell mass and glucose consumption and (b) biosurfactant production at varying initial glucose concentrations (10, 30, 50, and 70 g/L) using *Bacillus* sp. BMN 14.

production by BMN 27 showed higher dependence on cell growth than that by BMN 14. Hence, the strain BMN 14 was more capable of producing biosurfactant with glucose as substrate. This was further supported by the higher decrease in surface tension with BMN 14 (up to 27.2 mN/m) than with BMN 27 (up to 34.3 mN/m). No change in surface tension values was observed after 18 h. This can be possibly attributed to the formation of other metabolic products [29]; hence, these metabolic products did not affect the surface tension. Moreover, a change in surface tension was caused by interface saturation with the surfactant molecules [8]. Because of the best performance of *Bacillus* sp. BMN 14 observed here, the strain was then employed for further studies below.

3.2. Effect of various initial glucose concentrations

Fig. 2 shows the effect of initial glucose concentration on the kinetic profiles of cell mass, glucose consumption, biosurfactant, and surface tension during the fermentation process with the strain BMN 14. Overall, there was a gradual drop in glucose concentrations for all initial concentrations. Glucose was used for growth in the logarithmic phase at early 18 h, while the biosurfactant was substantially produced after 12 h. It seemed that the growth of the strain was more inhibited at an initial glucose concentration of 70 g/L, which could be related to the osmotic phenomenon at high concentration [15]. This consequently affected the production of biosurfactant; a lower biosurfactant concentration was observed with an initial glucose concentration of 70 g/L compared to 50 g/L glucose. Higher initial glucose concentration led to lower glucose consumption at the end of fermentation, and this incomplete consumption of glucose may have resulted in the inhibition of the produced biosurfactant [30].

Table 1 presents the summary of the effect of initial concentration of glucose on cell mass yield, biosurfactant productivity, biosurfactant yield, and surface tension with the strain BMN 14. The cell mass decreased by about twice when the glucose concentration was increased from 10 to 30 g/L. Cell mass yield continued to decline with increasing initial glucose concentration because of substrate inhibition [30]. Biosurfactant yield and productivity decreased substantially when the glucose concentration was increased from 50 to 70 g/L. The highest biosurfactant production and productivity were observed at a glucose concentration of 50 g/L. This initial glucose concentration resulted in the smallest value of surface tension at the end of fermentation. The low values of surface tension obtained here were in accordance with the values of surface tension in the literature for *Bacillus* sp. strains [4,5,7].

Table 2 presents the CMC for biosurfactant and the surface tension for various strains of *Bacillus* sp. A similar surface tension was observed between the surfactin in this work and the surfactin in the standard sample (from Sigma-Aldrich). The values of surface tension obtained from the literatures in the range of 27–42 mN/m were also comparable to those obtained in this work (10.1–38.5 mg/L; Table 2). CMC values for surfactin in the literature are in the range of 10.2–63.0 mg/L. These variations in CMC values depend on the properties of the solvent used for dissolving the surfactin [31,32]. The purified surfactin from Sigma-Aldrich showed the most efficient performance, with a CMC value of 7.8 mg/L. The CMC values obtained here declined from 38.5 to 10.1 mg/L with glucose concentrations 50 and 70 g/L, respectively, and are in agreement to those obtained from the literature [3]. This finding is reasonable because the biosurfactant concentration reached its highest value at the cultivation time and higher surface tension for 70 g/L (29.8 mN/m) was also obtained compared to the value for 50 g/L (27.1 mN/m).

3.3. Effect of C/N ratio

Fig. 3 shows the effect of ammonium nitrate concentration on the kinetic profiles of cell mass, glucose consumption, and biosurfactant production during fermentation with the strain BMN 14. Table 3 presents the summary of the effect of ammonium nitrate concentration

Table 1
Effect of initial glucose concentration on the performance of *Bacillus* sp. BMN 14.

Initial glucose (g/L)	Cell mass yield[a] (g/g)	Biosurfactant productivity[a] [g/(L·h)]	Biosurfactant yield[a] (w/w)	Surface tension (mN/m)
10	0.364	0.070	0.316	27.4
30	0.198	0.064	0.150	28.7
50	0.135	0.077	0.091	27.1
70	0.113	0.031	0.035	29.8

[a] Calculated at maximum cell mass concentration and biosurfactant production.

Table 2
Minimum surface tension and CMC for various strains of *Bacillus* sp.

Surfactant	Surface tension (mN/m)	CMC (mg/L)	Reference
S0 = 10 g/L for strain of BMN 14	27.4	16.0	This work
S0 = 30 g/L for strain of BMN 14	28.7	32.0	This work
S0 = 50 g/L for strain of BMN 14	27.1	38.5	This work
S0 = 70 g/L for strain of BMN 14	29.8	10.1	This work
SDS (synthetic)	37.0	2.9	[36]
Surfactin from *B. subtilis* LAMI005	30–35	10–63	[3]
Surfactin from *B. subtilis* isolate BS5	42.5	15.6	[8]
Surfactin from *B. subtilis*	27.0	25.0	[37]
Surfactin from *B. pumilus* 2IR	31.0	30.0	[9]
Standard surfactant (Sigma-Aldrich)	27.0	7.8–21	[3]

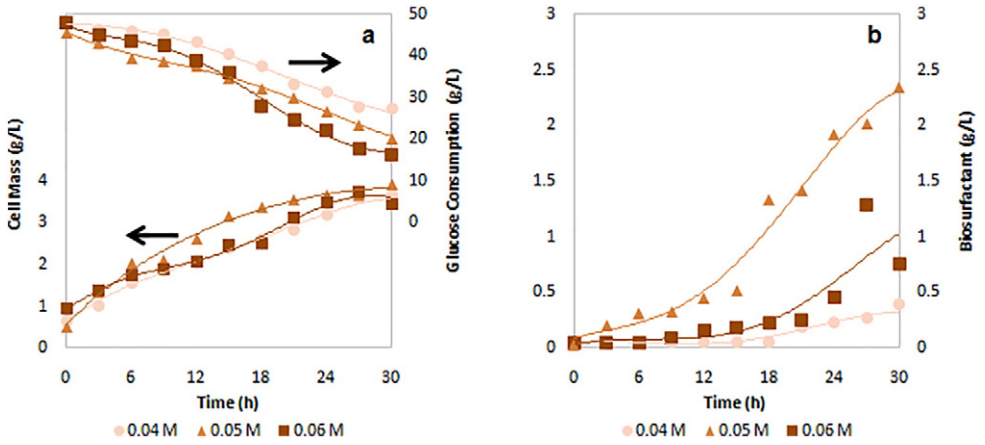

Fig. 3. Profiles of (a) cell mass and glucose consumption and (b) biosurfactant production at varying ammonium nitrate concentrations (0.04, 0.05, and 0.06 M) using *Bacillus* sp. BMN 14.

on cell mass yield, biosurfactant productivity, biosurfactant yield, and surface tension. The addition of ammonium nitrate at 0.04, 0.05, and 0.06 M led to C/N ratios of 17.51, 12.36, and 10.56, respectively, as shown in Table 4. Higher values of nitrogen source resulted in more consumption of glucose, as observed in Fig. 3. However, optimum biosurfactant production was obtained at the C/N ratio of 12.36, at which the biosurfactant productivity and yield were 0.077 g/(L·h) and 0.091%, respectively. The lowest surface tension (27.05 mN/m) was also observed at this ratio. It is possible that nitrogen source at a certain amount resulted in considerable performance of the strain [13]. As shown in Table 4, the medium with the initial C/N ratio of 12.36 showed the highest decrease in the C/N ratio (34% decrease) at the end of the fermentation compared to the decrease observed with other ratios. This implies that the amount of nitrogen consumed at this ratio was higher than the amount of carbon consumed compared to other ratios; hence, BMN 14 actively produced the biosurfactant. This finding can be attributed to the affinity permease by nitrogen concentration [33].

3.4. Kinetic model for cell mass, biosurfactant productivity, and surface tension

Kinetic models are useful tools that are applied for further process expansion and/or industrial implementation [21]. The modified Gompertz kinetic model is generally used for predicting the ethanol production [21,34] and predicting the cumulative biohydrogen production [35]; in this study, it was applied for predicting the biosurfactant production. The modified Gompertz model used is defined according to the equation below:

$$\gamma_i = \gamma_{i,m} \exp\left[-\exp\left[\frac{r_{i,m} \cdot \exp(1)}{\gamma_{i,m}}(t_{L,i}-t)+1\right]\right] \quad \text{[Equation 1]}$$

where i denotes cell mass (Cell) or biosurfactant (Sur). For cell mass, γ_{Cell} represents the concentration of cell mass (g/L), $\gamma_{Cell,m}$ is the potential maximum concentration of cell mass (g/L), $r_{Cell,m}$ is the maximum cell mass formation rate [g/(L·h)], and $t_{L,Cell}$ is time to exponential cell mass formation or lag phase (h). For biosurfactant, γ_{Sur} is biosurfactant concentration (g/L), $\gamma_{Sur,m}$ is the initial cell mass concentration (g/L), $r_{Sur,m}$ is the maximum biosurfactant rate [g/(L·h)] and $t_{L,Sur}$ is time to exponential biosurfactant production or lag phase (h).

Table 3
Effect of addition of nitrogen source on the performance of *Bacillus* sp. BMN 14.

NH_4NO_3 concentration (M)	Cell mass yield[a] (g/g)	Biosurfactant productivity[a] [g/(L·h)]	Biosurfactant yield[a] (w/w)	Surface tension (mN/m)
0.04	0.148	0.012	0.018	31.06
0.05	0.135	0.077	0.091	27.05
0.06	0.080	0.024	0.023	28.00

[a] Calculated at maximum cell mass concentration and biosurfactant production.

Table 4
Effect of ammonium nitrate concentration on carbon to nitrogen ratio.

Parameter	NH_4NO_3		
	0.04 M	0.05 M	0.06 M
C/N at the beginning	17.51	12.36	10.56
C/N at the end	6.87	8.11	3.52
Decrease in C/N at the end (%)	60.76	34.39	66.61

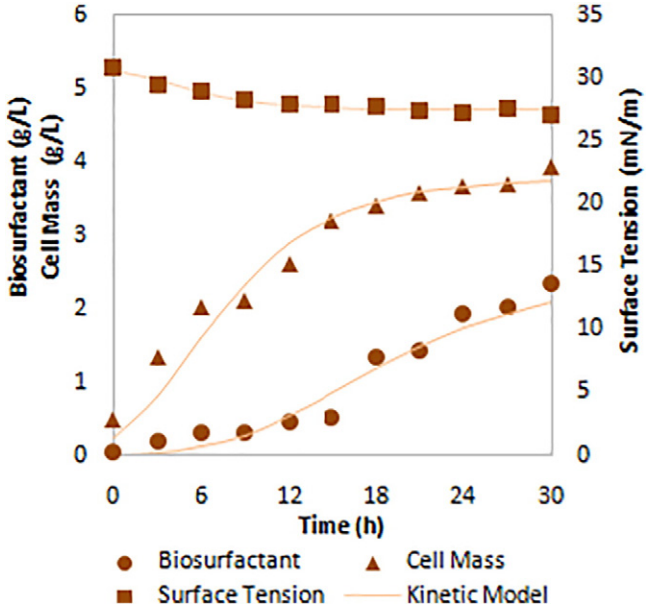

Fig. 4. Kinetic model prediction against experimental data for cell mass, biosurfactant production, and surface tension.

Table 5
Estimated parameters of cell mass, biosurfactant production, and surface tension with the modified Gompertz kinetic model.

Estimated parameters											
Cell mass				Biosurfactant production				Surface tension			
$\gamma_{Cell,m}$ (g/L)	$r_{Cell,m}$ [g/(L·h)]	$t_{L,Cell}$ (h)	R^2	$\gamma_{Sur,m}$ (g/L)	$r_{Sur,m}$ [g/(L·h)]	$t_{L,Sur}$ (h)	R^2	$\eta_{Sten,m}$ (mN/m)	$r_{Sten,m}$ [mN/(m·h)]	$t_{L,Sten}$ (h)	R^2
3.752	0.27	0.16	0.95	2.46	0.11	7.2	0.95	-3.41	-0.32	0.01	0.99

The modified Gompertz equation was further extended to enable the prediction of the surface tension in broth media as follows:

$$\eta_{Sten} = \eta_{Sten,0} + \left(\eta_{Sten,m}\right) \exp\left[-\exp\left[\frac{r_{Sten,m} \cdot \exp(1)}{\left(\eta_{Sten,m}\right)}\left(t_{L,Sten}-t\right)+1\right]\right] \quad \text{[Equation 2]}$$

where η_{Sten} is the surface tension (mN/m), $\eta_{Sten,0}$ is the initial surface tension (mN/m), $\eta_{Sten,m}$ is the potential drop in surface tension (mN/m), $r_{Frcts,m}$ is the maximum surface tension loss rate [mN/(m·h)], and t_L is time to the exponential drop in surface tension (h).

Fig. 4 shows the comparison between the experimental data and model predictions for cell mass, biosurfactant, and surface tension. A good fitting for the prediction of cell mass and biosurfactant production was observed with the coefficients of regression (R^2) of 0.95 and 0.95, respectively, as shown in Table 5. The lag phase value of $t_{L,Cell}$ (0.16 h) showed that the cell grew immediately at the beginning of the process. $t_{L,Sur}$ for surfactant produced (7.2 h) was evaluated in accordance with the experimental value. The value of $\gamma_{Sur,m}$ predicted the maximum produced biosurfactant concentration, which was 2.46 g/L and was in agreement with those in literatures using various *Bacillus* sp. [3,8]. The negative signals on $\eta_{Sten,m}$ and $r_{Sten,m}$ indicated the potential drop in surface tension and the loss rate for the maximum surface tension, respectively. The prediction of the surface tension using the expansion of the modified Gompertz kinetic model excellently fitted the experimental data (Fig. 4), with a coefficient of regression (R^2) of 0.99 (Table 5).

4. Conclusions

At initial glucose concentrations of 10–70 g/L, *Bacillus* sp. BMN 14 produced biosurfactant concentrations of 1–2.46 g/L. The bacteria showed good performance, with a decrease in surface tension up to 27 mN/m. The C/N ratio of 12.4 was an optimum condition for the production of biosurfactant and resulted in the highest decrease in surface tension. The cell mass and biosurfactant concentrations could be well predicted using the modified Gompertz equation. The extended modified Gompertz equation very well predicted the surface tension.

Conflict of interest

The authors declare no conflict of interest.

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