Investigation of fermentation conditions for *Candida bombicola* ACTT22214 from molasses and soybean oil for sophorolipid production

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ABSTRACT

Sophorolipid (SL) is a biosurfactant belonging to the glycolipids group and was produced by harmless *Candida bombicola* ACTT22214 and has been widely used in many fields in our life. In order to search for appropriate condition for *C. bombicola* fermentation producing SL with high efficiency, this study focused on the investigation of the soy oil concentration, molasses concentration, fermentation time, pH and temperature. SL products were tested for antimicrobial activity, antioxidant, emulsifier, foaming ability. The highest content of SL was 43.27 ± 0.30 g/L under conditions of: soybean oil content 5%, molasses content 150 g/L, fermentation time 7 days, pH = 5, 28^0C fermentation temperature. The antibacterial activity of SL was good: the highest resistance to *Candida albicans* (16.33 ± 1.15 mm), good resistance to *Bacillus spizizenii* (13.67 ± 0.58 mm), resistance to *Staphylococcus aureus* (12.67 ± 1.15 mm), relatively weak resistance to *Pseudomonas aeruginosa* (11.33 ± 0.58 mm) and *Escherichia coli* (9.67 ± 0.58 mm). The antioxidant capacity of SL was quite high with an IC_{50} value of 6.024 mg/mL. The emulsifying capacity of SL was equivalent to the emulsification of the tween 20 at a concentration of 5 – 10 mg/mL. SL had the ability to foam evenly from concentrations of 5 to 20 mg/mL but not higher than the corresponding concentrations of tween 20, SL was smooth, even, stable longer than tween 20.

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1. Introduction

Surfactants are bipolar compounds reducing surface tension between liquids with liquids, solids or gases, therefore they are able to combine and dissolve in water or other liquids. Most used surfactants are originally from petroleum and chemically synthesized, these compounds are environmental hazards because of their low biodegradability and high toxicity when dissolved in water (Bogaert, 2008). Thus, searching for replaceable biological surfactants is a necessary strategy. Microbiologically synthesized surfactants, including sophorolipid produced by fermentation of *C. bombicola* are considered due to sophorolipid application and commercialization potential, and significantly because sophorolipid are produced by non-pathogenic and safe *C. bombicola* (Bogaert et al., 2011) which performs high productivity (Dubey et al., 2013).

Sophorolipid are glycolipid biological surfactants, they are bipolar molecules formed by one disaccharide sophorose group bonding hydroxyl group of the second-to-last carbon atom in the

C16 - C18 fatty acid chain. For the production of sophorolipid by the fermentation of *C. bombicola*, two main substrates are provided: hydrophiliccarbon source: glucose and hydrophobic carbon source: fatty acids, oil, fatty acid ester, alkan. There are two types of sophorolipid: free acid form and lactone ring (Bogaert et al., 2007). This difference results in distinction in sophorolipid physical and chemical characteristics, acidic sophorolipid show foaming ability and high solubility, otherwise, sophorolipid with lactone rings show antibacterial activity and reduce surface tension effectively.

Sophorolipid are used in food, medicine, cosmetics, detergents (Bogaert et al., 2007). Recent research provided some particular applications of SL. Sophorolipid are used in washing powder as a detergent (Gobbert et al., 1984). Sophorolipid emulsification is utilized in petrochemistry. They are used in recovery of secondary petroleum products, removing hydrocarbons in crude petroleum. Sophorolipid are used in treating hydrocarbon contaminated soil and water, absorbing heavy metals in sediment and improve the quality of flour in food industry (Gobbert et al., 1984; Mager et al., 1987; Daniel et al., 1998). Sophorolipid perform antibacterial activity in pimples , dandruff and body's smell treatment, protect skin and hair, stimulate metabolism of epithelial fibroblast cells and synthesis of collagen for skin (Gorin et al., 1961; Cooper & Paddock, 1984). Sophorolipid also inhibit free radicals and aging elastase enzyme, promoting skin healing and whitening (Isoda et al., 1997). Diacetyl lactone SL can kill carcinoma cell lines such as liver cancer cell line H7402, decrease mortality rate due to septic shock in lab rats (Kim et al., 2005; Daverey & Pakshirajan, 2009), inhibit the development of leukemia cells (Spencer et al., 1970).

For industrial production and commercialization, SL must be competitive with chemical surfactant in 3 main perspectives: cost, uses and yield. Therefore, searching for low-cost material and setting up procedure for producing SL effectively are important. This study investigates appropriate conditions for *C. bombicola* fermentation in producing SL from molasses and soybean oil, in addition to conducting test of physical, chemical and biological characteristics of obtained SL.

2. Material and Methods

2.1. Material

Lyophilized *C. bombicola* ATCC 22214 was provided by professor Kim Eun-Ki, Inha University, South Korea. Strain was grown in YM Broth (glucose 1%, yeast extract 0.6%, peptone 0.5%); 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1',4"-sophorolactone 6',6"-diacetate were provided by Sigma (St. Louis, USA). Organic solvent: methanol, ethyl aceate, petroliumether were provided by Xilong company (China). Soy oil content 89.9% (Simply brand) was produced by Cai Lan vegetable oil company; molasses content 55% were provided by Kim Minh company; tested bacterial strains were provided by the Research Center of Bioactive Natural Products – University of Science, Ho Chi Minh City.

2.2. Methods

2.2.1. Propagation of *Candida bombicola* before fermentation

Lyophilized *C. bombicola* was propagated in YM media, after 48 hours, primary culture was sub cultured, producing secondary culture; *C. bombicola* propagation conditions include: temperature of 28^0C and shaking speed at 180 rpm in 48 hours. Secondary culture was used for fermentation and experiments.

2.2.2. Experiments for investigating appropriate conditions for *Candida bombicola* fermentation producing sophorolipid

Five one-factor experiments were randomly designed to investigate conditions including: molasses content, soybean oil content, temperature, pH and fermentation time (sophorolipid obtaining time) (Figure 1). Media used in these experiments contain: yeast extract 0.5%; KH_2PO_4 0.1%; $MgSO_4.7H_2O$ 0.05%, $CaCl_2.2H_2O$ 0.01%; NaCl 0.01%; peptone 0.07%. These experiments were conducted under conditions of shaking-speed at 180 rpm and fermentation medium was inoculated with 5% (v/v) seed medium. Erlenmeyer flasks (250 mL) with 50 mL of media were used in the above experiments. Crude sophorolipid yield was observed.

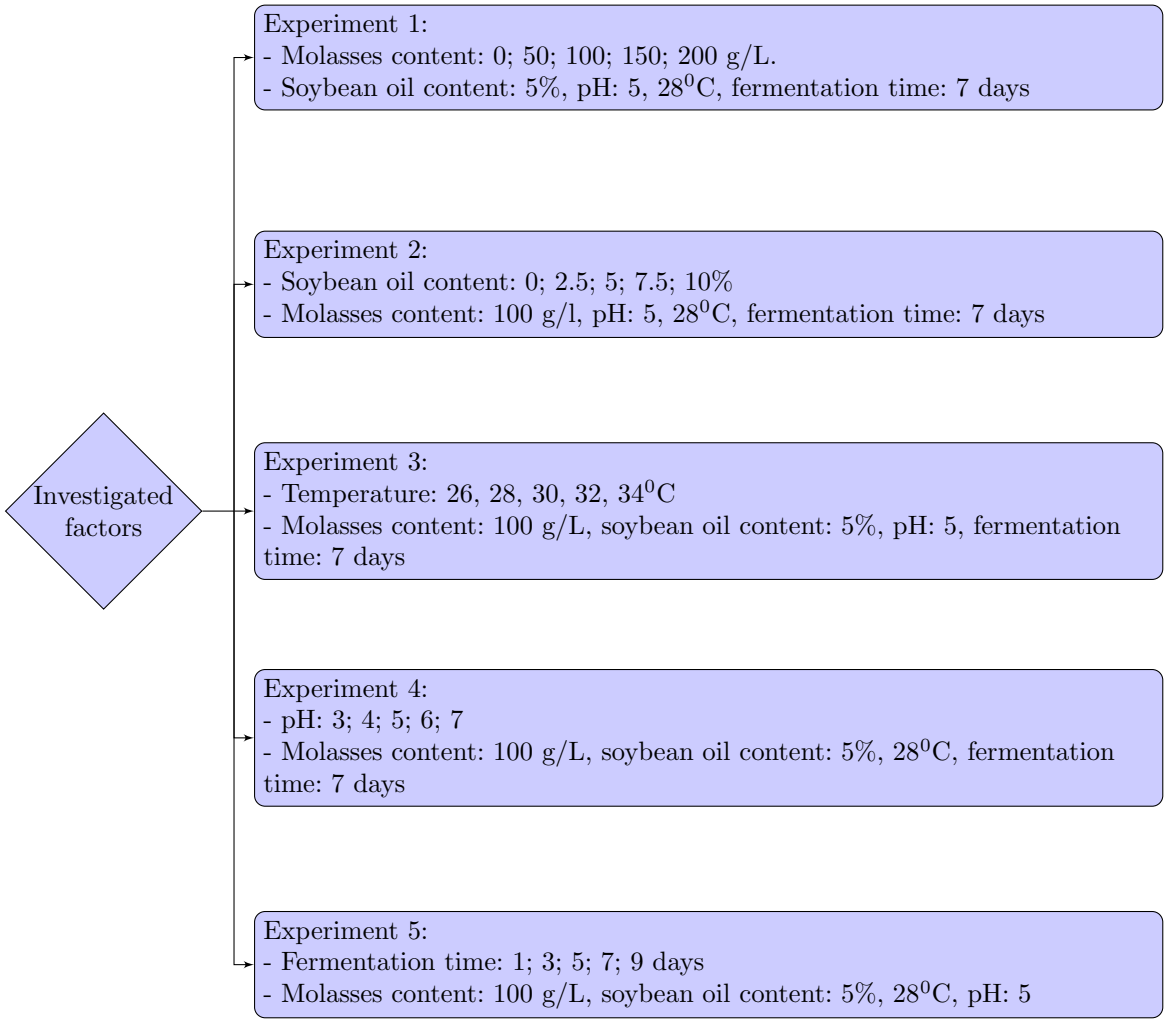

Figure 1. Experiments for investigating appropriate conditions for *C. bombicola* fermentation producing sophorolipid.

2.3. Sophorolipid separation from fermented broth and analysis of obtained SL by thin layer chromatogrphy TLC

2.3.1. Sophorolipid separation from fermented broth

Ethyl acetate (EtAc) was added into culture broth (1:1 v/v, twice), then centrifuged (6,000 rpm in 5 minutes), supernatant was collected and vacuum dried in rotary evaporatorat 40^0C to remove EtAc. After that, Petroliumether: Methanol (1:1 v/v, twice) was added and collect the lower layer (crude SL and methanol), then vacuum dried in rotary evaporator at 40^0C to remove methanol, crude SL was weighed (Figure 2).

2.3.2. Analysis of obtained SL by thin layer chromatogrphy TLC

Crude sophorolipid was spotted on chormatography plate then immersed in solvent system containedchloroform:methanol: H_2O (80:10:2 v/v/v) in 30 minutes. 1',4"-sophorolactone 6',6"-diacetate was used as the standard. After elution, the plate was sprayed with acid sulfuric 90% and dried at 100^0C, the spots were visualized.

2.4. Sophorolipid activity analysis

2.4.1. Emulsification test

Sophorolipid was diluted in DMSO 5% solution into different concentrations then add 5mL of each sample into test tubes (diameter: 1.6 cm, height: 16 cm). Add the same volume of diesel into these tubes then vortex in 2 minutes, leave for 10 minutes and observe emulsification after 10 minutes, 12 hours and 24 hours, measure the height of emulsified layer, emulsification index after 24 hours was calculated as the formula: E24 = (height of emulsified layer/total height) x 100.

2.4.2. Foaming ability of SL

Sophorolipid was diluted in DMSO 5% solution into different concentrations. After that, add the same volume of 10 mL of each sample into test tubes (diameter: 1.6 cm, height: 16 cm), tight the caps and shake the tubes vertically in 1 minute then leave them for a while. Foaming was observed, and height of foam layers was measured after shaking and after leaving for 5 minutes. Repeat the same procedure or the control (tween 20), the experiment was repeated 3 times.

2.4.3. Antibacterial activity

Testing of antibacterial activity was conducted using agar diffusion method. Bacterial strains: *Escherichia coli*, *Staphylococus aureus*, *Bacillus spizizenii*, *Pseudomonas aeruginos* were inoculated in LB agar plates and Candida albicansin SD agar plates with paper disks were placed on, 20 μL SL (100 mg/mL) was added to paper disks and incubated 37^0C in 1 – 2 days. Inhibition zones were observed.

2.4.4. Free radical scavenging activity

Free radical scavenging activity was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. Crude SL was dissolved in methanol into different concentrations, 100 μL of each sample was added into 96-well plate, 100 μL DPPH 300 μM was then added and mixed. The plate was incubated at 37^0C in 30 minutes, then OD was measured at 517 nm wavelength. The percentage of free radical scavenging was calculated as the formula: % antioxidant = (1 - OD sample/OD control) x 100.

3. Results and Discussion

3.1. Morphology of *C. bombicola*

C. bombicola was recovered and grown in YM media after 2-day inoculation at 28^0C. Culture was diluted into concentration of 10^{-9} and inoculated on YM agar plates, the colonies showed creamy color, smooth and glistening dome shape with entire margin, the diameter was from 0.4 - 0.7 cm. Observing under microscope using 100X objective lens showed the elongated oval shape of *C. bombicola* and its budding reproduction (Figure 3).

3.2. Testing of appropriate conditions for *C. bombicola* fermentation producing SL

Recovered *C. bombicola* was grown in YM media in 2 days at 28^0C, with 180 rpm of shaking speed to obtain the concentration of 109 CFU/mL. This culture was used as seed medium for below experiments.

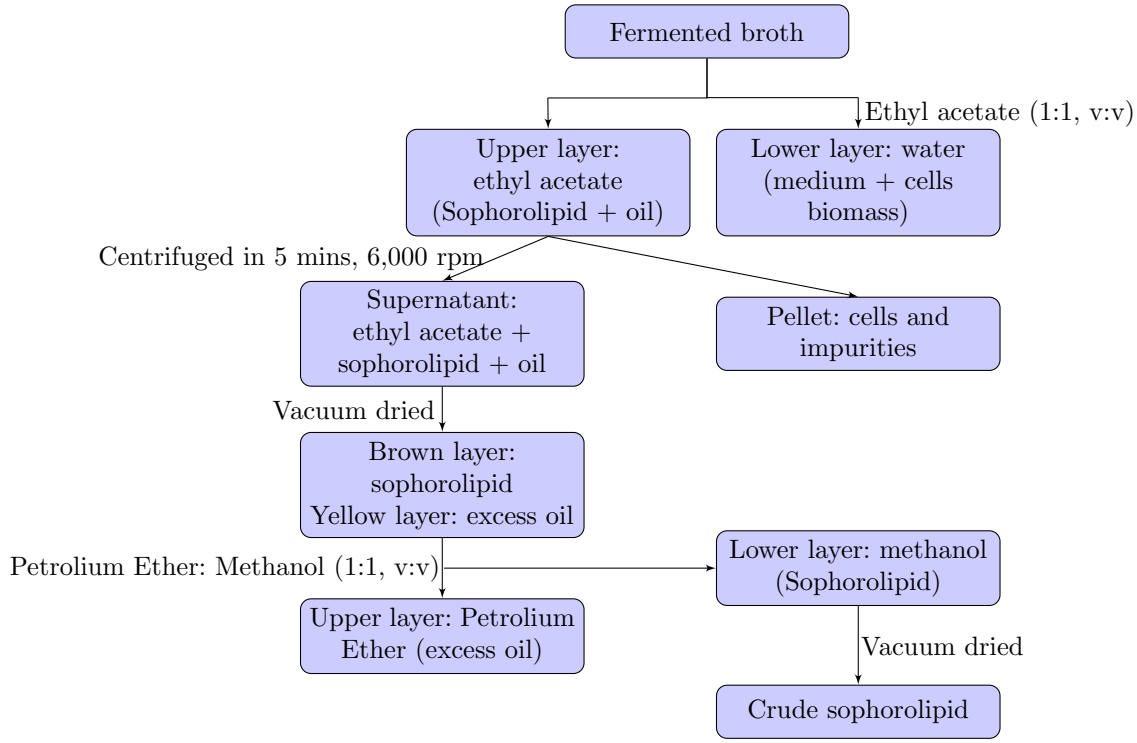

Figure 2. Procedure of separating sophorolipid from fermented broth.

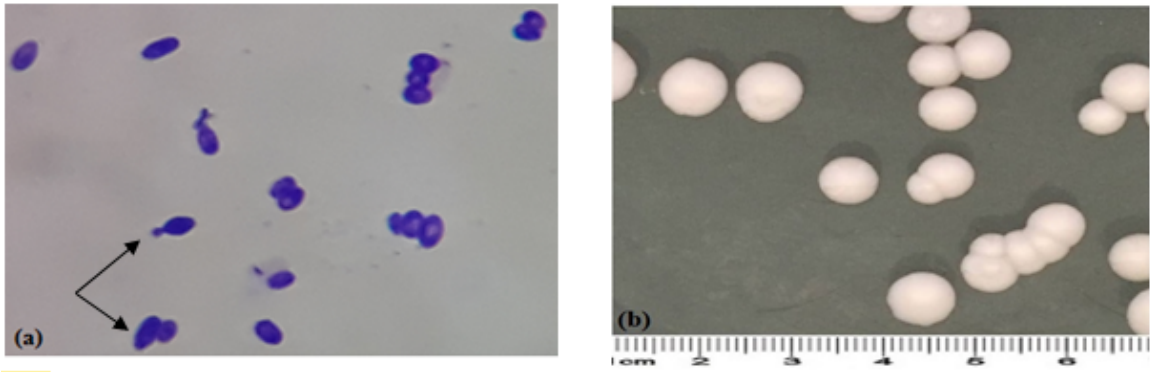

Figure 3. Morphology of yeast *C. bombicola*. (a) *C. bombicola* observed under microscope with 100X objective lens shows oval shape and budding reproduction, (b) *C. bombicola* colonies on YM agar plates after 48-hour incubation at 28^0C.

3.2.1. Investigation of molasses content affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples containing molasses contents of 0; 50; 100; 150, 200 g/L. The result showed that the highest SL production of C. bombicola was 43.27 ± 0.31 g/L when the fermentation media was composed of 150 g/L. The results were shown the difference from the media composed of 100 g/L was not significant. When increasing the molasses concentration to 200 g/L, SL production decreased. It was probably due to the high osmosis pressure caused by the high concentration. Therefore, the growth and development of *C. bombicola* were affected in the adapting stage (Table 1).

Table 1. Investigation of molasses content affecting the fermentation of *C. bombicola* producing SL

Treatments	Molasses content (g/L)					
	0	50	100	150	200	
Sophorolipid (g/L)	$0.33^{d} \pm 0.12$	$33.33^{c} \pm 0.50$	$41.87^{a} \pm 0.64$	$43.27^{a} \pm 0.31$	$38.33^{b} \pm 0.83$	

[a-d] Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

3.2.2. Investigation of soybean content affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples containing soybean contents of 0; 2.5; 5.0; 7.5, 10%. The results showed the highest yield of SL was 42.27 ± 0.31 g/L at 5% of soybean content. However, there was no significant difference from the media containing the soy oil concentration of 7.5%. On the other hand, when increasing the amount of soy oil to 10%, SL production decreased significantly. This was probably due to the lower density of soy oil compared to water and its indissolubility in water. Therefore, the high concentration disrupted the diffusion of oxygen in the media (Table 2).

3.2.3. Investigation of temperature affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation at the temperature 26; 28; 30; 32; 34^0C. The results showed the highest yield of SL was 41.87 ± 0.53 g/L at the temperature of 28^0C. At 34^0C, SL yield was nearly none because *C. bombicola* was almost unable to grow so the production of SL was stopped (Table 3).

3.2.4. Investigation of pH affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation at pH conditions: 3; 4; 5; 6; 7. The results showed the highest yield of SL was 42.07 ± 0.53 g/L at pH = 5. We found that there was no significant difference from the result when pH = 6 (Table 4).

3.2.5. Investigation of product obtaining time affecting the fermentation of *C. bombicola* producing SL

C. bombicola was respectively inoculated into testing samples for fermentation with obtaining time 1; 3; 5; 7; 9 fermenting days. The result showed the highest SL concentration (41.67 ± 0,61 g/L) when the obtaining time was after 7 days. The SL production decreased significantly after 9 days of producing because when the substrate became exhausted, *C. bombicola* would use SL as a nutrient source. Therefore, the in-

Table 3. Investigation of temperature affecting the fermentation of *C. bombicola* producing SL

Treatments	Temperature (^{0}C)				
	26	28	30	32	34
Sophorolipid (g/L)	$38.40^{b} \pm 0.53$	$41.87^{a} \pm 0.31$	$41.00^{a} \pm 0.72$	$36.53^{c} \pm 0.61$	$0.20^{d} \pm 0.00$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Table 2. Investigation of soy content affecting the fermentation of *C. bombicola* producing SL

Treatments	Soybean content (%)				
	0.0	2.5	5.0	7.5	10
Sophorolipid (g/L)	$13.40^{d} \pm 0.53$	$31.20^{c} \pm 0.72$	$42.27^{a} \pm 0.42$	$41.00^{a} \pm 0.20$	$38.53^{b} \pm 0.61$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Table 4. Investigation of pH affecting the fermentation of *C. bombicola* producing SL

Treatments	pH					
	3	4	5	6	7
Sophorolipid (g/L)	$38.87^{c} \pm 0.31$	$40.13^{bc} \pm 0.42$	$42.07^{a} \pm 0.46$	$41.33^{ab} \pm 0.61$	$37.13^{d} \pm 0.76$

[a-d]Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

appropriate SL obtaining time decreased the SL amount significantly (Table 5).

Therefore, after the above experiments, the appropriate conditions for *C. bombicola* fermentation producing SL were shown: 150 g/L of molasses content, 5% of soybean oil content, 28^0C, pH = 5, obtaining time is after 7 days of fermentation. From the above data, fermentation of *C. bombicola* was conducted, the yield was 43.27 ± 0.30 g/L. SL obtained is brown,viscous liquid (Figure 4).

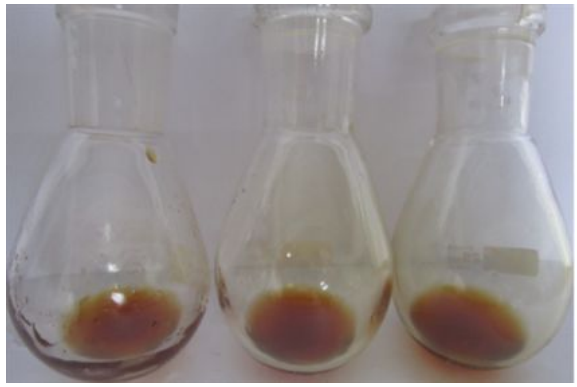

Figure 4. Obtained SL from the fermentaion of *C. bombicola.*

According to Table 6, SL yield was considered high (43.27 g/L), higher than results in the research of Asmer et al. (1988), Nguyen et al. (2017) and Le et al. (2016) which were 34; 21.8 and 14.6 g/L, respectively. However, SL yield was lower than in research of Cooper & Paddock (1984), Deshpande & Daniels (1995), and Zhou et al. (1992) which were 68; 97 and 138 g/L, respectively. The difference in SL yield can be due to different source of substrates, fermenting conditions as well as the time of obtaining SL. Therefore, it is important to have further research on optimal conditions as well as appropriate substrate contents for the fermentation.

3.3. Analysis of obtained SL by thin layer chromatogrphy TLC

Figure 5 shows the existance of 1',4"- sophorolactone 6',6"-diacetate in crude SL, proving the suitability of crude SL extraction in obtaining SL from fermented broth. In addition, visualized spots were at different positions showing different structures of obtained SL.

Table 6. Comparison of SL production reported by others to our result

Media components (g/L)	Time (h)	Max level of SL (g/L)	References
Glucose: 100; oleic: 36; yeast extract: 10	120	34.00	Asmer et al. (1988)
Glucose: 100; sunflower oil: 95; yeast extract: 5	100	68.00	Cooper & Paddock (1984)
Glucose: 100; safflower oil:105	154	138.00	Zhou et al. (1992)
Glucose: 100; catfish fat: 100 yeast extract: 5	168	21.80	Nguyen et al. (2017)
Glucose: 100; coconut oil: 100; yeast extract: 5	168	14.60	Le et al. (2016)
Glucose: 100; fat: 100	60	97.00	Milind Deshpande & Lacy Daniels (1995)
Molasses: 150, soybean oil: 50; yeast extract: 5	168	43.27	This work

Table 5. Investigation of product obtaining time affecting the fermentation of *C. bombicola* producing SL

Treatments	Product obtaining time (days)				
	1	3	5	7	9
Sophorolipid (g/L)	$0.27^{d} \pm 0.12$	$5.40^{c} \pm 1.25$	$35.53^{b} \pm 1.10$	$41.67^{a} \pm 0.61$	$35.27^{b} \pm 0.90$

$^{a\text{-}d}$Average values followed by letters were statistically significant difference by $LSD_{0.01}$ test.

Figure 5. Chromatogram of SL analysis obtained SL; (C) standard substance (1',4"-sophorolactone 6',6"-diacetate), the Rf value is 0.55.

3.4. Testing of physical, chemical and biological of SL

3.4.1. Foaming ability of SL

To determine the foaming ability of SL, shake test tubes containing SL and 20 tween (control) dilutled in DMSO 5% solution into different concentrations were shaked vertically in 1 minute, observing and measure the height of foam layer twice: after shaking and after leaving for 5 minutes.

Based on Figure 6, the foaming ability of the two surfactants SL and tween 20 increased as their concentrations increased. The amount of foam produced by tween 20 was much higher than by SL. After 5 minutes, the amount of foam formed by the two surfactants was reduced but negligible, the foam volume was maintained quite well. At the same time, smooth, uniform, and stable foam produced by SL was formed, relatively durable compared to the tween 20.

3.4.2. Testing the emulsifying ability of SL

Determination of emulsifying ability of SL was carried out by uniformly vortexing SL and tween 20 solutions diluted in 5% DMSO solution into different concentrations in 2 minutes, leave them to stand still and observe the emulsion. After 10 minutes, 12 hours and after 24 hours, measure the height of the emulsified layer, determine the E24 index of solutions, with E24 being the emulsifying percentage after 24 hours.

Based on Figure 7, the emulsification of both SL and tween 20 increased as the concentration increased. However, at the concentration of 20 mg/mL, the emulsification of SL is very low, almost none; while at concentrations of 5 mg/mL and 10 mg/mL, the emulsification of SL is almost equivalent to tween 20. The decrease or loss of emulsification capacity when the concentration of SL increases is due to SL's tendency of aggregate into large masses, its contact and dispersion into the water phase and the oil phase decreases, sometimes the accumulation was too much, the SL mass becomes heavier, leading to deposition to the bottom and making the emulsification not good.

3.4.3. Examination of antibacterial activity of SL

In order to determine the antibacterial activity of SL, aspirate 20 μL of 100 mg/mL SL solution onto paper plates placed on the surface of agar containing the tested microorganisms. Positive antibiotics are gentamicin (100 μg/ml) or terbinafine (100 μg/mL) and negative methanol (90%).

Based on Figure 8 and Table 7, it shows that SL is most resistant to *C. albicans*, followed by *B. spizizenii*, then *S. aureus*, and finally weak resistance to *P. aeruginosa* and *E. coli*. Thus, it is possible to conclude that SL is more resistant to Gram (+) bacteria than Gram (-) bacteria. This finding is consistent with researches the research of Kim et al. (2005). The resistance to *C. albicans* which is a fungus parasitic on human body of SL proves that SL has the potential to be used as safe detergent and antiseptic.

Table 7. Results of the ring diameter of sophorolipid resistance for microorganisms

Microorganisms	Antibacterial ring diameter of SL (mm)
B. spizizenii	13.68 ± 0.58
C. albicans	16.33 ± 1.15
E. coli	09.67 ± 0.58
P. aeruginosa	11.33 ± 0.58
S. aureus	12.67 ± 1.15

3.4.4. Investigation of the antioxidant capacity of sophorolipid by DPPH (1,1-Diphenyl-2-picrylhydrazyl)

Add 100 μl of each SL solutions at different concentrations into 96 well microplates. Af-

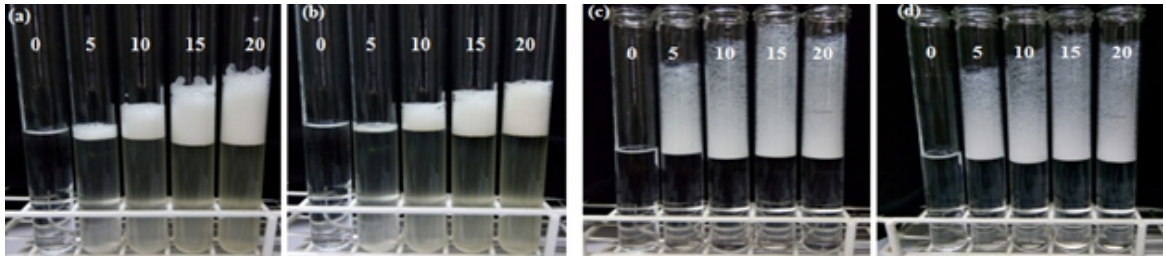

Figure 6. Results of the foaming test of SL and tween 20. (a) SL after shaking, (b) SL after leaving for 5 minutes, (c) Tween 20 after shaking, (d) Tween 20 after leaving for 5 minutes. SL/tween 20 concentrations were 0; 5; 10; 15; 20 (mg/mL).

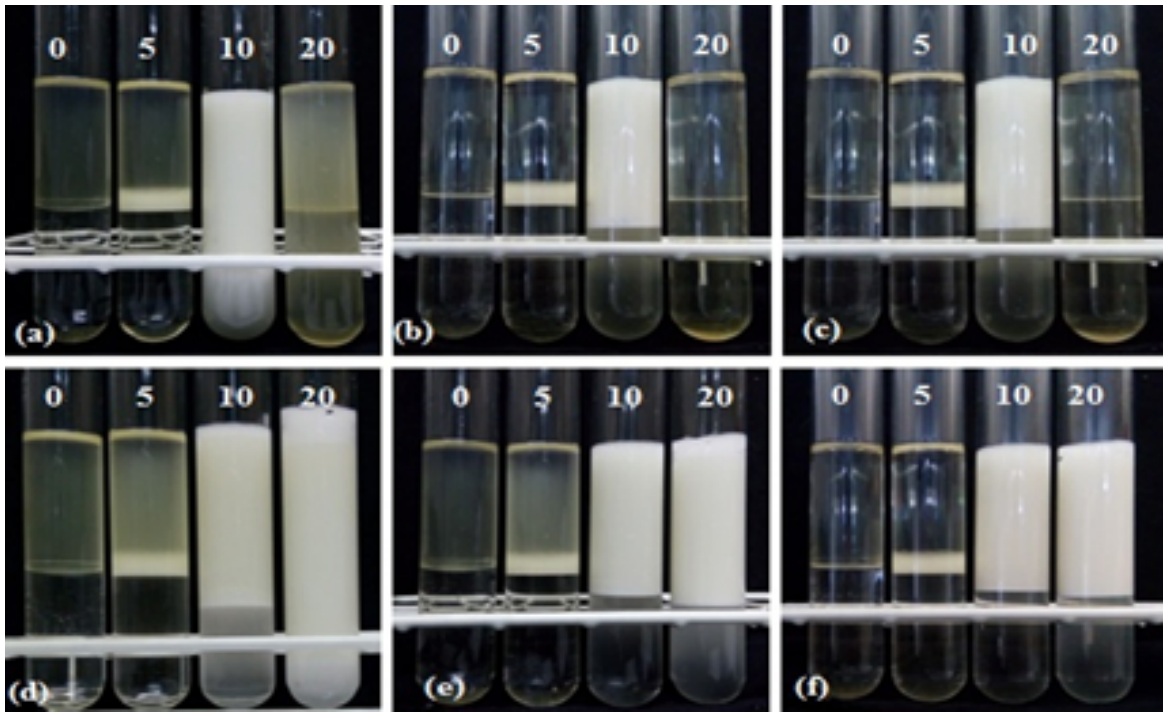

Figure 7. Results from emulsion investigations of SL and tween 20. (a), (b), (c) emulsification of SL after 20 minutes, 12 hours and 24 hours; (d), (e), (f) emulsification of tween 20 after 20 minutes, 12 hours and 24 hours. The investigated SL/tween 20 concentrations were 0; 5; 10; 20 (mg/mL).

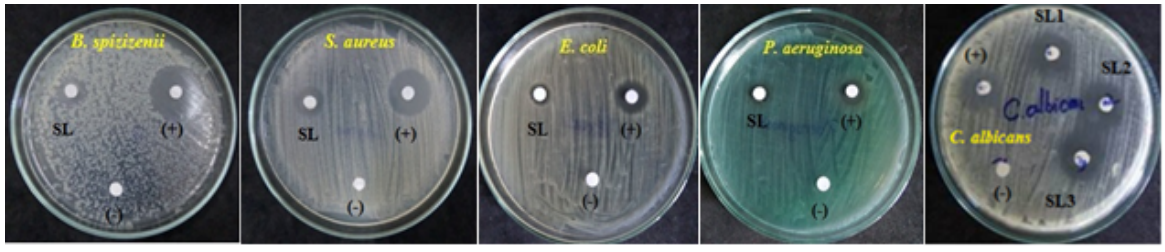

Figure 8. Antibacterial results of SL with paper disk method, (+): Positive test; (-): Negative test.

ter adding 100 μl DPPH, incubate at 37^0C for 30 minutes, then determine the color by ELISA reader and acknowledge the percentage of antioxidant corresponding to each concentration. Therefore, determination of the expressed curve shows the relationship between the substance concentration and the corresponding antioxidant percentage of SL.

From Figure 9, the IC_{50} value is 6.024 mg/mL. With IC_{50} value of 6.024 mg/mL, it can be seen that the antioxidant capacity of SL is quite good. This can be applied to produce useful antioxidants in cosmetic products such as skin lotion, aiming to care and protect the skin.

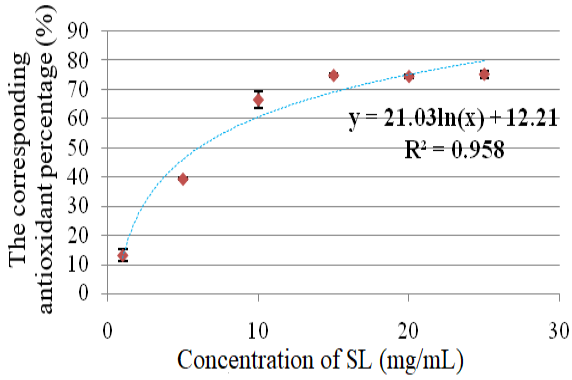

Figure 9. The graph shows the antioxidant percentages of SL by DPPH.

4. Conclusion

Initially, suitable conditions for fermentation of C. bombicola for SL production are found to be quite high at 43.27 g/L with the following parameters: 150 g/L of molasses; 5% of soybean oil, fermentation temperature is 28^0C, pH = 5, fermentation time is 7 days. SL product has good antimicrobial properties, antioxidant capacity with an IC_{50} of 6.024 mg/mL, the ability to foam, durable and stable emulsification equivalent to chemical surfactants. Therefore, it shows the high application potential of SL in this study in areas such as cosmetics and detergents.

Acknowledgements

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