Asia-Pacific Journal of Science and Technology: Volume: 22. Issue: 02. Article ID.: APST-22-02-06. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Faculty of Engineering, Khon Kaen University, Thailand

Production and characterization of sophorolipids produced by *Candida bombicola* using sugarcane molasses and coconut oil

Nguyen Luong Hieu Hoa,[1] Le Quynh Loan,[2] Kim Eun-Ki,[3] Tong Thu Ha,[4] Ngo Duc Duy,[2] Hoang Quoc Khanh,[2] Nguyen Hoang Dung[1,2,*]

[1] Nguyen Tat Thanh Hitech Institute, Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[2] Institute of Tropical Biology, Vietnam Academy of Science and Technology
[3] Inha University, Incheon, Korea
[4] Nong Lam University, Ho Chi Minh City, Vietnam
*Corresponding author: nhdung@ntt.edu.vn

Abstract

Sophorolipids (SLs) are a type of biosurfactant that belong to the glycolipids group and have many applications in various fields. They have been used to replace chemical surfactants due to their higher biodegradability, lower toxicity, and their being more environmentally friendly. However, in order to compete with cheaper chemical surfactants, it is important to use suitable low-cost materials for sophorolipid production. In this study, we produced SLs from *Candida bombicola* using sugarcane molasses and coconut oil. The results showed that the optimal conditions for SL production were 10% (w/v) sugarcane molasses and 10% (v/v) coconut oil at 25ºC and pH 6 for seven days. The sophorolipids that were produced were resistant to *E. coli* NRRL B-409, *P. aeruginosa* NRRL B-14781, *S. aureus* NRRL B-313, and *B. subtilis* NRRL B-354. They also exhibited free radical scavenging ability in a dose-dependent manner (EC_{50} = 0.35 mg/ml) and emulsification activity. These results showed that sugarcane molasses and coconut oil can be used for SL production in order to reduce substrate costs.

Keywords: *Candida bombicola*, sophorolipids, sugarcane molasses, coconut oil, biosurfactant

1. Introduction

Surfactants are one of the most important classes of industrial chemicals, with about 15 million tons being produced per year worldwide [1]. However, almost all of the commercially available surfactants are derived from petroleum through chemical synthesis [2]. These compounds are less biodegradable and are often toxic to the environment. Their use may, thus, lead to significant ecological problems[3]. Surfactants of microbial origin have gained increasing attention due to their potential advantages over chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, and higher specific activity at extreme temperatures, pH, and salinity [4], [5] & [6].

Sophorolipids (SLs) are a kind of extracellular biosurfactant. They are composed of the disaccharide sophorose (2'-O-β-D-glucopyranosyl-β-D-glycopyranose), which is linked to a long fatty acid chain (C16-C18) [7]. They are synthesized by a selected number of yeasts species such as *Candida bombicola, C. magnolia, C. apicola*, and *C. bororiensis* [8], with *C. bombicola* being the most popular strain for SL production. Sophorolipids have two main structure groups: acid form (open structure) and lactone form (ring structure). Sophorolipids have many different applications such as their use as a cleaning agent. The emulsifying properties of SLs can be useful in secondary oil recovery and removing hydrocarbons from drill material. Sophorolipids also act as antibacterial agents in the treatment of acne, dandruff, and protection of hair and skin, as they stimulate dermal fibroblast metabolism and collagen synthesis. In addition, SLs also act as antifungal agents against fungal plants such as *Phytophthora* sp. *and Pythium* sp., antihuman immunodeficiency virus and exhibit a cytotoxic effect on several human cancer cell lines [1].

There have been some studies published from Vietnam that relate to the isolation and production of surfactants from some bacterial strains, almost all of which were isolated from polluted oil lands [9]. However, SLs have not been studied intensively enough. Sugarcane molasses is an abundant byproduct of the sugar industry but it has not been fully utilized in this regard. It is mostly used as animal feed, fertilizer, or in alcohol production. Most previous studies have used medium-chain fatty acids in SL production (C16-C18), but there have not been many studies on the use of short-chain fatty acids (C12-C14) for this purpose. In this study, we used sugarcane molasses as a low-cost fermentative hydrophilic carbon source to replace glucose and coconut oil as a short-chain carbon source for SL production and examined the properties of SLs produced by *C. bombicola* through fermentation.

2. Materials and methods

2.1 Materials

Professor Kim Eun-Ki of Inha University in Incheon, South Korea was kind enough to provide us with *the candida bombicola* ATCC 22214 yeast strain used in this study (1′,4″-Sophorolactone 6′,6″-diacetate and 2,2-diphenyl-1-picrylhydrazyl - DPPH [Sigma)]. The organic solvent (n-hexane, methanol, and ethyl acetate) was supplied by Xilong, China. Sugarcane molasses was supplied by the Bien Hoa Sugar Refinery, and coconut oil was supplied by the Thanh Vinh Company in Ben Tre, Vietnam.

Bacterial test strains *B. subtilis* NRRL B-354, *S. aureus* NRRL B-313, *P. aeruginosa* NRRL B-14781, and *E. coli* NRRL B-409 were maintained on LB agar slant at 4^{o}C.

2.2 Methods

2.2.1 Inoculum development

Freeze-dried *C. bombicola* ATCC 22214 was activated in an MGYP medium containing 0.3% malt extract, 1% glucose, 0.3% yeast extract, and 0.5% peptone for 48 hours at 25^{o}C, pH 6, and 180 rpm shaking. Inoculum was then subcultured and incubated a further for 48 hours.

2.2.2 Sophorolipids production

The fermentation medium contained 10% (v/v) coconut oil, 10% (w/v) sugarcane molasses, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2.2H_2O$, 0.01% NaCl, and 0.07% peptone. After that, 5% inoculum was added to 250 ml Erlenmeyer flasks containing 100 ml production medium, and the process of SL production began at 25^{o}C, pH 6, 180 rpm for seven days.

2.2.3 Effects of parameters on SL production

The effects of various factors, such as fermentation time, temperature, pH, and sugarcane molasses and coconut oil concentrations, on SL production were investigated. Samples were taken to analyze the biomass and SL concentrations.

2.2.4 Biomass estimation and SLs separation

The fermentation broth was centrifuged and the supernatant and cell pellet were then extracted with hexane to remove the remaining oil and other fatty acids. The next extracted with ethyl acetate to obtain SLs. The cell pellet was washed with distilled water and dried to a constant weight at 60^{o}C to determine its biomass. The ethyl acetate layer was vacuum dried at 40^{o}C to remove the solvent.

2.2.5 Analysis of SL composition by TLC and HPLC

The SL samples were dissolved in ethyl acetate and spotted on Silica gel plates. The plates were then immersed in solvent systems containing chloroform/methanol/H_2O (80:10:2 v/v/v). Once the solvent front had moved approximately 4/5 the height of the plates, they were removed, sprayed with H_2SO_4 40%, and dried at 100^{o}C until spots were observed [6].

For HPLC analysis, SLs were dissolved in methanol. The C_{18} Chrom Silicycle column (4.6×150 mm), 5 µm was used in the stationary phase; the mobile phase consisted of a mixture of methanol:water (gradient 30-100%) for 50 min, at a temperature of 25 ± 2^{o}C. The flow rate was 1 ml/min, the injection volume was 20 µl, and the UV detector wavelength was 210 nm.

2.2.6 Analysis of fatty acid composition by GC

The fatty acid composition of the SLs was analyzed using GC, which was provided by the Center of Analytical services and Experimentation in Ho Chi Minh City, Vietnam.

2.2.7 Antibacterial activity

Antibacterial activity was tested using a well diffusion method and determined diameter of the inhibition zone on the plates [10]. The MIC method was used to determine the minimum inhibitory concentration. The SL samples and bacterial cultures were added to a 96-well plate and incubated at 37^{o}C for 24 hours. The absorbance of the plates was measured at a wavelength of 610 nm (with Ampicilin as a positive control and Mueller Hinton medium as a negative control) [7]. The minimum inhibitory concentration is the concentration at which the growth of bacteria could not be observed.

2.2.8 Antioxidant activity

Antioxidant activity was determined using DPPH assay [8]. The SL sample and DPPH solution were added into a 96-well plate and then mixed. The plate was incubated at 37^{o}C for 30 min and the absorbance at the 517 nm wavelength was measured. Ethanol was used as a negative control and ascorbic acid was used as a positive control. The percentage of free radical scavenging was calculated using the formula: % antioxidant = (1 - OD sample/OD control)*100.

2.2.9 Emulsification activity

Emusification activity of the SLs was investigated by measuring the emulsifiction index E_{24} [4]. The SL samples were mixed with non-aqueous phase liquid (DO oil, hexane, rapeseed oil, soybean oil) in a vortex mixer for 2 min and allowed to stabilize for 24 hours. The emulsification index E_{24} was calculated using the formula: (the height of emulsification layer/ the height total of the solution)*100.

3. Results and discussion

3.1 Effects of various factors on SL production

The factors that were found to affect SL production are showed in Figure 1. The results showed that SL yield increased from the third day to the fifth day, and the maximum yield was obtained after seven days. The optimal temperature for SL production was observed at 25^{o}C. Optimal SL production was observed at pH 6, whereas above and below pH 6, both yield and biomass decreased. These results are similar to those of previous studies. Ashby *et a*l. [11], for example, also obtained maximum SLs at pH 6, 25^{o}C using oleic acid and glucose. A possible reason for this is that the enzymes involved in the cellular metabolism of SL formation become more active at this pH and temperature. In terms of substrate concentration, the highest of SL production was observed at 10% sugarcane molasses and coconut oil concentration. This affects SL production because the substrates are required for both the formation and cellular metabolism and of SLs. This finding was consistent with that of a previous studies by Daverey *et al*. [4] & Solaiman *et al.* [12].

3.2 Analysis of SL composition by TLC and HPLC

To determine whether or not the product obtained consisted of SLs, we analyzed and compared the samples with standard SLs 1′,4″-Sophorolactone 6′,6″-diacetate (Sigma) produced using TLC (Figure 2). The results confirmed the presence of 1′,4″-Sophorolactone 6′,6″-diacetate in the SL samples. In addition, various forms of SLs were detected. The SL samples were then analyzed using HPLC (Figure 3). The chromatogram showed more than 10 peaks. Four major peaks (5, 6, 7, and 8) were eluted from 29 min to 32 min. The peak detected at 31 min was the most prominent. Retention time of the peaks was similar those found in previous reports, Morya et al. [8], for example, analyzed SLs produced from sugarcane molasses and oleic acid and found peaks from 16 to 35 min, with the two peaks at retention times 26 and 31 min identified as diacetylated lactonic SLs C18:1 and C18:2. Gupta et al. [6] also identified diacetylated lactonic SLs at 27 min, 31 min, 32 min, and 34 min, as well as non-acetylated lactonic SLs at 18 and 21 min.

Figure 2 Thin layer chromatography plate of SLs: 2,3: SLs sample, C: SLs standard (1′,4″-Sophorolactone 6′,6″-diacetate).

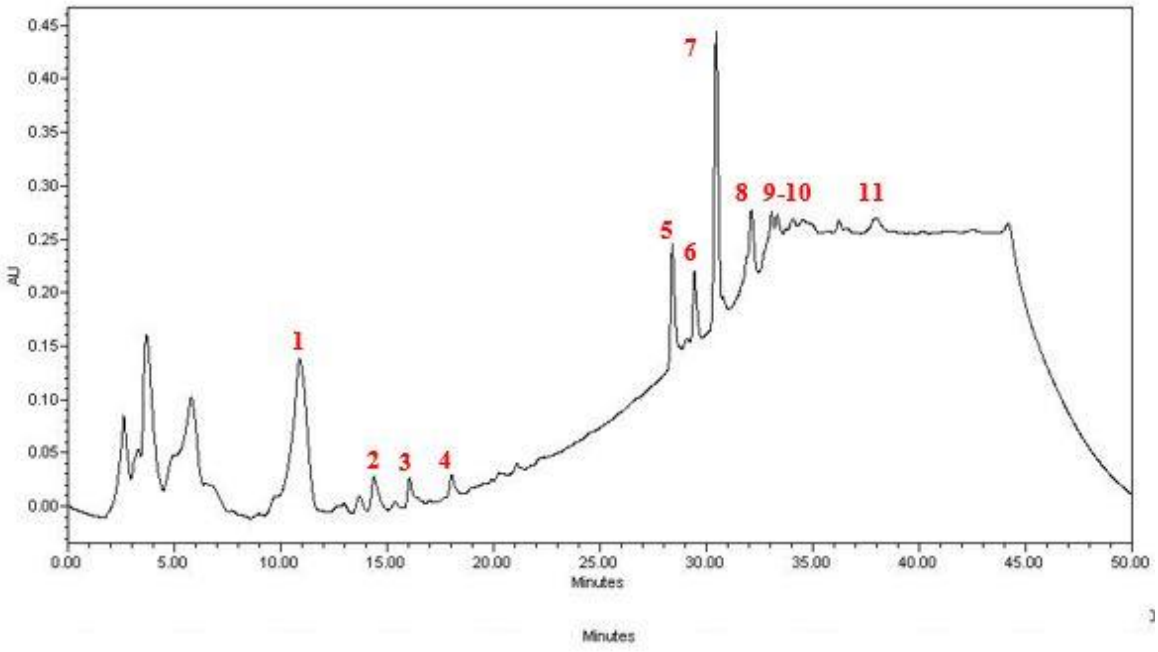


Figure 3 HPLC chromatogram of SLs.

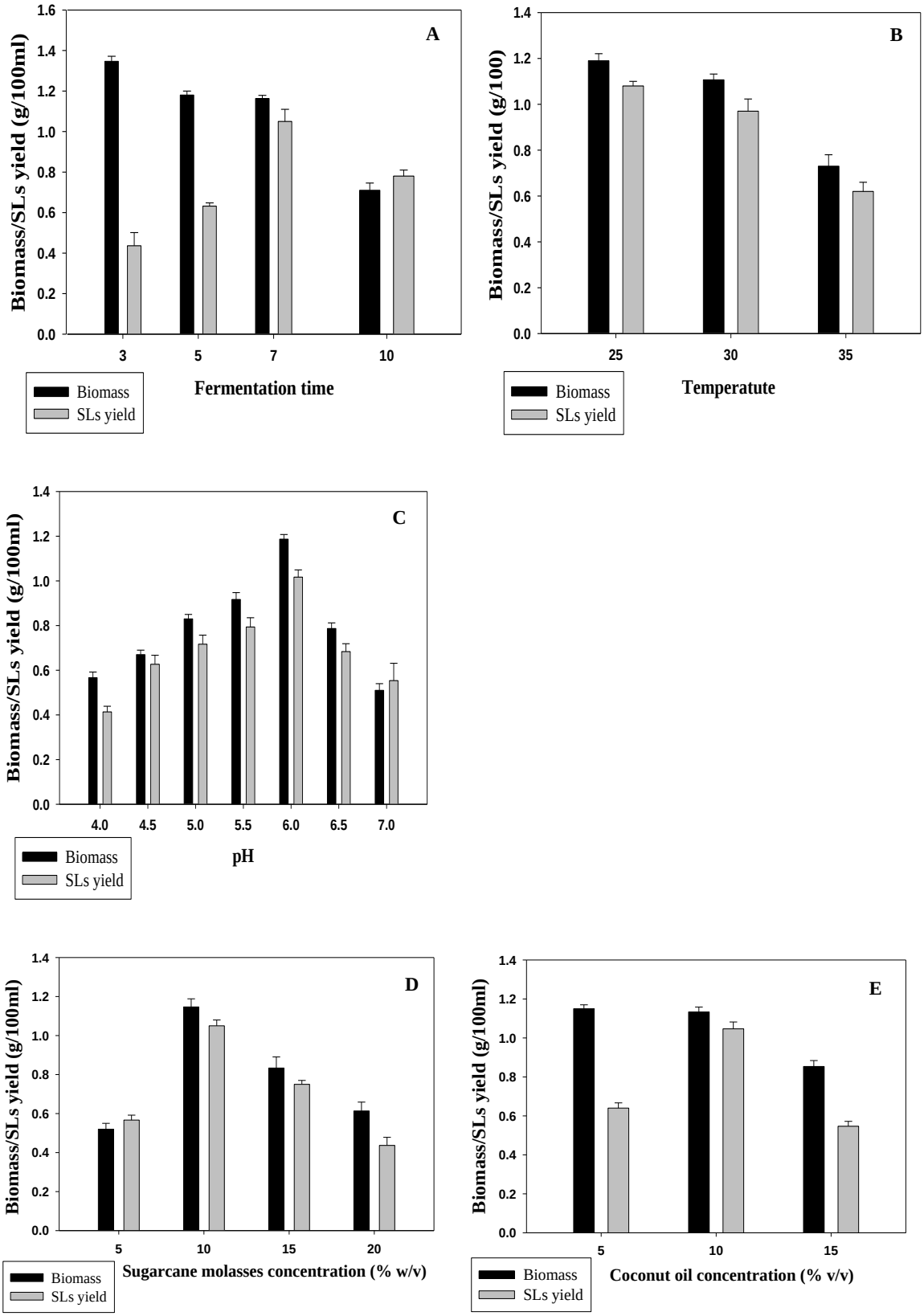


Figure 1 Effect of various factors on SLs production. A: Fermentation time, B: Temperature, C: pH, D: sugarcane molasses concentration and E: coconut oil concentration.

3.3 Analysis fatty acid composition of SLs sample by GC

Fatty acid composition in the SL samples were anlayzed using the GC method. The results showed that the major of fatty acid chains present in the samples were C12 and C14; C12:0 (26.61%), C14:0 (51.50%). Medium- and long-chain fatty acid (C16, C18 and C20) were also observed but the composition was quite low. This demonstrates the tendency of direct incorporation of fatty acids. Coconut oil is known to be one of the richest sources of medium-chain fatty acids, such as lauric acid and myristic acid. Previous studies have also shown the similar fatty acid composition when using soybean oil and rapeseed oil. Develter and Lauryssen [4], for example, analyzed the fatty acid composition of SLs produced using glucose and soybean oil and found a C18:1 fatty acid composition of 26.45%, C18:2 composition of 60.88%. In addition, Daniel *et al*. [13] found that the C18:1 and C18:2 fatty acid composition of SLs produced by whey and rapeseed oil were 50.7% and 26.38%, respectively.

3.4 The antibacterial activity of SLs

The findings with regard to antibacterial activity are summarized in Table 1 and Figure 4. The results showed that SLs exhibited stronger antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. Although the antibacterial activity of SLS in this study was low when compared with previous studies [10] & [8], this results still showed the potential applications of SLs as an antiseptic for cleaning fruits and vegetables, or to be used in combination with antibiotics to improve treatment.

3.5 The antioxidant activity of SLs

The free-radical scavenging ability of the SLs was analyzed using DPPH assay (Figure 5). The results showed that SLs were able to scavenge free radical molecules in a dose-dependent manner (EC_{50} was 0.35mg/ml). The antioxidant and antibacterial activity of SLs suggest their potential applications in cosmetics and pharmaceuticals.

Table 1 Antibacterial activity of SLs.

	Bacterial strains	Diameter of the inhibition zone (cm)	MIC (mg/ml)
1	*Staphylococcus aureus*	2.15 ± 0.21	4.5
2	*Bacillus subtilis*	1.40 ± 0.10	5.0
3	*Escherichia coli*	1.10 ± 0.05	10.0
4	*Pseudomonas aeruginosa*	1.10 ± 0.03	10.0

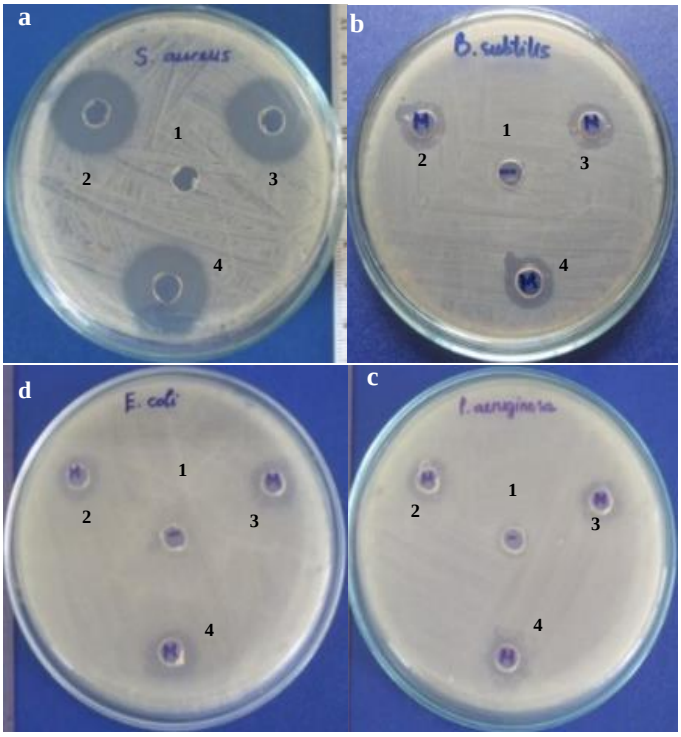


Figure 4 Antibacerial activity of SLs. a: *S. aureus*; b: *B. subtilis*, c: *P. aeruginosa*; d: *E. coli*,1: negative control, 2,3,4: SLs samples.

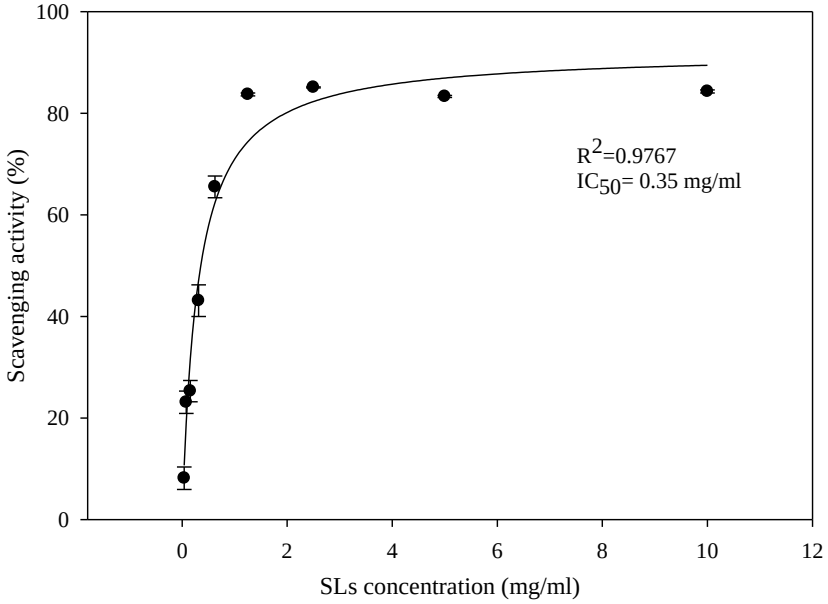
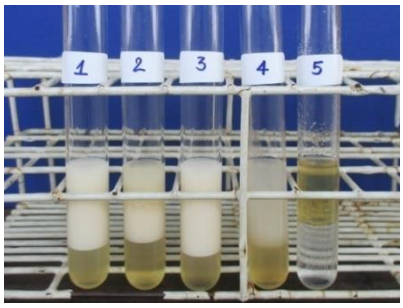


Figure 5 The ability to scavenge free-radical molecules of SLs.



Substrate	Emulsification index (E_{24}) (%)
Hexane	62.5 ± 0.89
DO oil	59.23 ± 0.51
Rapeseed oil	65.18 ± 0.89
Soybean oil	69.64 ± 0.90
H_2O	00.00 ± 0.00

Figure 6 Emulsification activity of SLs. 1: rapeseed oil, 2: DO oil, 3: soybean oil, 4: hexane, 5: H_2O (negative control).

3.6 The emulsification activity of SLs

The emulsification activity of SLs is showed in figure 6. At 30ºC and after 24 hours, the SL samples were capable of emulsifying all of the substrate. Among the four substrates tested, SLs exhibited the strongest emulsification activity with soybean and rapeseed oil and weakest activity with hexane and DO oil. This result is consistent with those of a study by Daverey et al. [14], which found that that SLs produced by *C. bombicola* using sugarcane molasses and rapeseed oil exhibited better emulsification activity and stability with diesel oil, benzene, xylene, and hexadecane.

5. Conclusion

In this study, we produced SLs from *C. bombicola* using sugarcane molasses as a hydrophilic carbon source as an alternative to costly glucose. The results showed that the optimal conditions for maximum SL yield was seven days of fermentation, 25ºC, pH 6, 10% (w/v) sugarcane molasses concentration, and 10% (v/v) coconut oil concentration. The SLs exhibited activity against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis*. SLs also exhibited emulsification activity and the antioxidant activity with EC_{50} at 0.35 mg/ml. These results suggested that SLs could be produced using sugarcane molasses to replace glucose, and that these SLs could be used in health care products and cosmetics.

6. References

[1] Shao, L., Song, X., Ma, J., L,i H., Qu, Y., 2012. Bioactivities of sophorolipid with different structures against human esophageal cancer cells. Journal of Surgical Research 173, 286-91.

[2] Van-Bogaert, I.N., Zhang, J., Soetaert, W., 2011. Microbial synthesis of sophorolipids. Process Biochemistry 46, 821-833.
[3] Desai, J.D., Banat, I.M., 1997. Microbial production of surfactants and their commercial potential. Microbiology and Molecular Biology Reviews 61, 47-64.
[4] Develter, D., Lauryssen, L.M.L., 2010. Properties and industrial applications of sophorolipids. European Journal of Lipid Science and Technology 112, 628-638.
[5] Ganesh, L.M., Parag, R.G., Aniruddha, B.P., 2015. Improved synthesis of sophorolipids from waste cooking oil using fed batch approach in the presence of ultrasound. Chemical Engineering Journal 263, 479-487.
[6] Gupta, R., Prabhune, A., 2012. Structural determination and chemical esterification of the sophorolipids produced by Candida bombicola grown on glucose and α-linolenic acid. Biotechnology Letters 34, 701-707.
[7] Kim, H.S., Kim, Y.B., Lee, B.S., Kim, E.K., 2005. Sophorolipids production by Candida bombicola ATCC 22214 from a corn-oil processing byproduct. Journal of Microbiology and Biotechnology 15, 55-58.
[8] Morya, V.K., Park, J., Kim, T.J., Jeon, S., Kim, E.K., 2013. Production and characterization of low molecular weight sophorolipid under fed-batch culture. Bioresource Technology 143, 282-288.
[9] Van, N.H., Nguyen, H.B., Dang, T.C.H., 2004. The ability to produce surfactant of KC31 bacteria strain. Journal of Biotechnology 2, 501-510 (In Vietnamese)
[10] Shah, V., Badia, D., Ratsep, P., 2007. Sophorolipids having enhanced antibacterial activity. Antimicrobial Agents and Chemotherapy 51, 397-400.
[11] Ashby, R.D., Nunez, A., Solaiman, D.K.Y., Foglia, T.A., 2005. Sophorolipid biosynthesis from a biodiesel co-product stream. Journal of the American Oil Chemists' Society 82, 625-630.
[12] Solaiman, D.K.Y., Ashby, R.D., Nunez, A., Foglia, T.A., 2004. Production of sophorolipids by Candida bombicola grown on sugar molasses as substrate. Biotechnology Letters 26, 1241-1245.
[13] Daniel, H.J., Reuss, M., Syldatk, C., 1998. Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage fed batch process using Candida bombicola ATCC 22214 and Cryptococcus curvatus ATCC 20509. Biotechnology Letters 20, 1153-1156.
[14] Daverey, A., Pakshirajan, K., 2010. Kinetics of growth and enhanced sophorolipid by Candida bombicola using a low-cost fermentative medium. Applied Biochemistry and Biotechnology 160, 2090-2101.