Screening and Characterization of Bacterial Biosurfactant from Bangkok and Vicinities

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Abstract—**In this study, biosurfactant producing bacteria were screened from oil-contaminated areas in Bangkok and vicinities, Thailand. Isolated bacteria of 1,094 isolates were collected from the samples. Primary screening of biosurfactant producing bacteria was performed by oil agar plate method. From primary screening, 82 isolates showed dispersion zone surrounding colonies. Ten isolates with the highest RZC values were selected to study in the secondary screening. These isolates were separately cultured in NB. Tensiometer was used to measure surface tension of each cell free cultured broth. The decreasing of surface tension was observed in 4 isolates. BKG 04-005 isolate showed the lowest surface tension value of 18.58mN/m. According to the results, biosurfactant of BKG 04-005 was growth-associated product. To obtain biosurfactant, its cell free cultured broth was acidified and extracted by acid and a mixture of chloroform: methanol, respectively. The chemical structure of crude biosurfactant was identified as a lipopeptide by FTIR spectroscopy.**

Index Terms—**screening, bacterial biosurfactant, surface tension**

I. Introduction

Surfactants are amphipathic molecules that composed of hydrophobic and hydrophilic moieties [1]. According to their chemical structures, surfactants are able to reduce surface and interfacial tensions of two immiscible phases. Chemical synthetic surfactants have been commercially used in industries [2]-[4]. For examples, surfactants are widely applied to lower the interfacial tension of crude oil and matrix substances in Oil-Enhanced Recovery (OER) in petroleum industry. Sodium Dodecyl Sulfate (SDS), an anionic surfactant, is used as a preoperative skin cleaner, bacteriostatic agent, and also in medicated shampoo [5]. Quaternary ammonium-based surfactants (QACs) are used as softener and antiseptic agents in fabric industry [6]. However, the disadvantages of synthetic surfactants have been reported such as environment pollution, animal and human toxicity [7], [8]. According to their disadvantages of chemical synthetic surfactants, biosurfactants are the interesting substances.

Biosurfactants are natural surface active compounds that produced by many kinds of living organisms, especially microorganisms [9]. Biosurfactant have recently been recognized as important microbial products with properties applicable in a number of industries and processes. Being capable of lowing surface and interfacial tensions, biosurfactants are today thought to be efficient replacers of chemically synthesized surface active agents. Some of their superior properties, such as biodegradability, less toxicity, and their specificity, make these microbial products both attractive for specific industries and environmentally acceptable [10]. Surfactin, is one of the most powerful biosurfactants. The natural form of surfactin was discovered in culture media of Bacillus subtilis. Purified surfactin was able to lower surface tension of water from 72.0mN/m to 27.0mN/m [11].

Biosurfactants are classified into 4 groups based on their chemical structures; glycolipids, lipopeptides, phospholipids, and polymeric biosurfactants [12]. Biosurfactants are attractive products for use in agricultural, environmental, pharmaceutical, biomedical, cosmetic and food applications [13]. Biosurfactants are able to efficiently use in handing industrial emulsions, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil [14]. Accordingly, the screening for new bacteria has been intensified.

In this study, biosurfactant producing bacteria were screened from oil-contaminated areas in Bangkok and vicinities, Thailand. Surface-active activity was investigated by surface tension measurement. Crude biosurfactant of an isolated strain with the lowest surface tension was extracted, and then identified its chemical structure by Fourier Transform Infrared (FTIR) spectroscopy.

II. Material and Methods

A. Sampling Areas

Biosurfactant producing bacteria were screened from oil contaminated samples; coconut milk shops, edible oil

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retailer shops and garages in Bangkok and vicinities. The abbreviations of sampling areas were shown in Table I.

TABLE I. OIL CONTAMINATED AREAS AND THEIR ABBREVIATIONS

Sampling areas	Abbreviations		
	Coconut milk shops	Edible-oil retailer shops	Garages
Arunummarin market, Bangkoknoi, Bangkok	PC	PO	PG
Bangkrapi market, Bangkrapi, Bangkok	BC	BO	BG
Bangkae market, Bangkae, Bangkok	BKC	BKO	BKG
Klongteoy market, Klongtoey, Bangkok	KC	KO	KG
Minburi market, Minburi, Bangkok	MC	MO	MG
Ortorkormarket, Jatujak, Bangkok	OC	OO	OG
Pakkred market. Nontaburi	NC	NO	NG
Sumrong market, Samutprakarn	SC	SO	SG
Thai market, Prathumthani	TC	TO	TG
Yingchareun market, Bangkaen, Bangkok	YC	YO	YG

B. Microorganisms and Cultured Condition

The screened isolates were storage at -18 ℃ in nutrient broth (NB; Difco, USA) containing 40% glycerol solution. To microorganism enrichment, each isolate was streak onto nutrient agar (NA; Difco, USA), and incubated at 30 ℃ for 48h. The isolates were then cultured in NB.

C. Screening of Biosurfactant Producing Bacteria

1) Bacterial isolation

Oil-contaminated samples were collected from Bangkok and vicinity. Each sample was collected by cotton swab technique. The cotton swab was enriched with NB and incubated at 30 ℃ for 48h. Mix culture was isolated by Ten-fold dilution technique. Bacteria with different morphology were collected and given their codes for the further experiments.

2) Primary screening of biosurfactant producing bacteria

Primary screening was performed by oil agar plate technique. This method was modified from Morikawa *et al.*, [15]. Single colony of each strain was spotted onto NA plate spreading with 60 μl auto-lubricant oil (Yamaha, Japan). Positive result was appearance of clear zone around colony after incubation at 30 ℃ for 48h, as shown in Fig. 1. Ten isolates with the highest of ratio of oil dispersion zone diameter to colony diameter (RZC) values were selected for secondary screening.

3) Secondary screening of biosurfactant producing bacteria

Each isolate of 10 selected isolates was cultured in NB at 30 ℃, 50rpm for 48h. Cultured broth was centrifuged at 4,000g, 4 ℃ for 15 min to collect Cell Free Culture Broth (CFCB). Forty milliliters of CFCB were measured surface tension by tensiometer K6 (Kruss, Germany) with Du-Nouy Ring method by triplicate experiments. Sterile NB was used as a control.

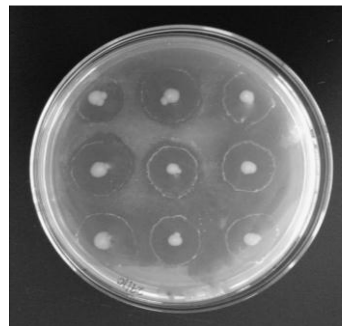

Figure 1. The example of positive result; the oil dispersion zones surrounding colonies

D. Growth Curve Investigation

Selected isolate was cultured in NB at 30 ℃, 100rpm for 60h. Cultured broth was collected every 6h for monitoring viable cell count. Briefly, viable cells of bacteria were elucidated by viable plate count technique with 10-fold serial dilution. The viable cells were investigated of dilution factor with 10^{-1} to 10^{-7}. Normal Saline Solution (NSS) with 0.85% NaCl was applied as diluent. One hundred microliters of sample was spread on NA plate and incubated at 30 ℃ for 48h. The surface tension of CFCB was measured during cultivation. The microbial growth and surface tension monitoring were performed with the triplicate experiments.

E. Biosurfactant Extraction

The selected strain was cultured in 1 liter NB at 30 ℃, 50rpm for 72h. The cultured broth was centrifuged at 4,000g, 4 ℃ for 15 min. CFCB was acidified to be pH 2 by 12 N HCl and incubated at room temperature for 24 h. Acidified CFCB was centrifuged at 15,000g to collect precipitate. The precipitate was re-dissolved in sterile water pH 7. Biosurfactant solution was then extracted 3 times by mixture solvent of chloroform:methanol (65:15). Organic phase was collected and evaporated by vacuum rotary evaporator to dryness. Crude biosurfactant was confirmed the surface activity by oil displacement technique [15].

F. Chemical Identification of Biosurfactant

The crude biosurfactant was identified their functional groups by FTIR spectroscopy (Perkin Elmer, USA). FTIR spectrum was carried out in transmittance mode by using $4cm^{-1}$ of resolution with the wave number range of 400 to $4,000cm^{-1}$. A lipopeptide biosurfactant, Surfactin (Sigma-Aldrich, USA) was used as a standard biosurfactant.

III. RESULTS AND DISCUSSIONS

A. Screening of Biosurfactant Producing Bacteria

Bacteria were obtained from 90 samples of oil-contaminated areas in Bangkok and vicinity. Isolated bacteria of 1,094 isolates were collected from the samples. Eighty-two isolates showed positive results in primary screening. Ten isolates with the highest of RZC were

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shown in Table II. After measuring surface tension of CFCB, 4 isolates showed their surface tension values lower than control; NB (52.53mN/m). BKG 04-005 showed the lowest surface tension of 18.58mN/m. This isolate was obtained from soil slurry sample of garage in Bangkae district, Bangkok. The decreasing of surface tension of CFCB was reported by many researchers. CFCB of Pseudomonas sp. 2B was lowered to 30.14mN/m [16]. The surface tension of water was reduced to 16.34mN/m by glycolipid producing from Nocardiopsis lucentensis [17]. Furthermore, lipopeptide deriving from Brevibacterium aureum MSA13 decreased the surface tension of water to 28.56mN/m [18]. The reduction of surface tension resulted from the amphipathic molecules in CFCB which accumulated at the interfaces of two immiscible phases (air-water interfaces) [4].

For 6 isolates of BKO 02-004, PG 04-001, BKG 01-005, PG 01-010, PC 01-003, and NG 02-004 showed clear zones around their colonies on the primary screening, but their surface tension values were higher than control. It was explained by the bacterial metabolic products. Bacteria were able to produce the biopolymer metabolites resulting in the increasing of surface tension and viscosity [19].

The surface tension of BKG 04-005 was significantly decreased at the exponential growth phase (Fig. 2). According to this result, biosurfactant producing by BKG 04-005 was an extracellular product. These results corresponded to *Streptomyces* sp. B3 that produced an extracellular biosurfactant during exponential growth phase [20]. In addition, Ismail *et al.*, [21] also reported that surface tension of Bacillus sp. I-15 cultured medium was decreased on the logarithmic phase.

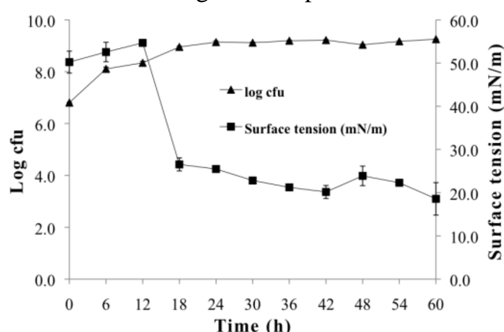

Figure 2. Growth curve and surface tension of BKG 04-005 isolate

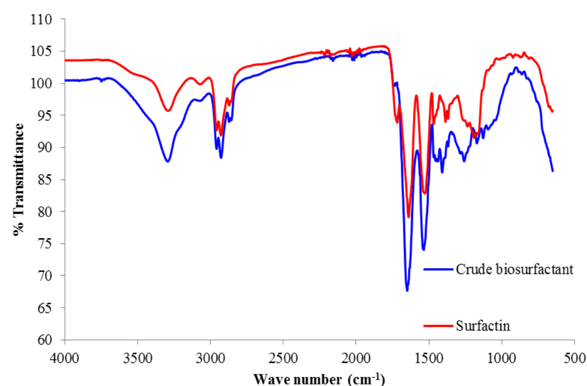

Figure 3. FTIR spectra of surfactin and BKG04-005 crude biosurfactant

B. *Biosurfactant Identification by FTIR*

After extraction, crude biosurfactant was recovered with a yield of 45.5mg/l. The dried crude biosurfactant was analyzed its chemical structure compared with Surfactin, a standard lipopeptide biosurfactant produced by *Bacillus subtilis*. The FTIR spectra of BKG 04-005 crude biosurfactant and Surfactin were shown in Fig. 3. The peak pattern of crude biosurfactants was similar to the FTIR spectrum of Surfactin. Board strong peak resulting from N-H stretching and CH-NH stretching were appeared at 3,281 and 1,649cm^{-1}, respectively. The peak at 2,924cm^{-1} was C-H stretching of aliphatic chain. These results were similar to lipopeptide biosurfactant from *Bacillus* subtilis 20B that showed three majors peaks of 3,305, 2,924 and 1,643cm^{-1}, which were interpreted to be peptide bond, aliphatic chain, and CH-NH, respectively [22]. Lipopeptide biosurfactant of *Bacillus licheniformis* F2.2 [23], *Bacillus subtilis* HOB2 [24], and *Sphingobacterium* sp. [25] also showed the similar pattern of FTIR spectrum of BKG 04-005 crude biosurfactant. It indicated that BKG 04-005 produced a lipopeptide biosurfactant.

TABLE II. RATIO OF OIL-DISPERSION DIAMETER PER COLONY DIAMETER (RZC) AND SURFACE TENSION OF CELL FREE CULTURED BROTH OF TEN ISOLATES

Isolates	RZC value	Surface tension (mN /m)
Control	-	52.53 ±1.60^{c}
BKG 04-005	4.81	18.58 ±3.75^{a}
NG 05-006	3.84	33.88 ±3.40^{b}
OC 01-004	3.84	33.93 ±5.71^{b}
PG 01-006	3.69	36.26 ±6.98^{b}
BKO 02-004	3.50	54.62 ±2.39^{cd}
PG 04-001	5.22	55.01 ±6.40^{cde}
BKG 01-005	6.26	58.37 ±4.66^{def}
PG 01-010	6.32	58.53 ±2.79^{def}
PC 01-003	5.46	58.55 ±3.50^{def}
NG 02-004	4.07	58.90 ±1.49^{def}

Different letter in the same column presented significant difference ($p \geq 0.05$).

IV. CONCLUSIONS

Oil contaminated samples were isolated bacteria of 1,094 isolates. Biosurfactant producing bacteria was screened by oil agar plate method in the primary screening. Eighty-two isolates showed the positive results by occurring oil dispersion zones surrounded their colonies in the primary screening. The RZC values were used as the criteria to select the bacterial isolates for the secondary screening. Ten isolates with the highest RZC values were investigated the surface tensions of their CFCBs in the secondary screening. For the secondary screening, BKG 04-005 showed the lowest surface tension of 18.58mN/m. While the surface tension of sterile NB, control, was 52.53mN/m. During cultivation, BKG 04-005 produced an extracellular biosurfactant. The FTIR spectrum indicated that biosurfactant of BKG 04-005 was lipopeptide. The further study, BKG 04-005 will be bacterial identified, and its biosurfactant will be studied on biological activity.

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REFERENCES

[1] M. T. Rosen and J. T. Kunjappu, *Surfactants and Interfacial Phenomena*, 4th ed., N. J. Hoboken: John Wiley and Sons, 2012, ch. 1, pp. 3-8.

[2] C. N. Mulligan and B. F. Gibbs, “Factors influencing the economics of biosurfactants,” in *Biosurfactants, Production, Properties, Applications*, N. Kosaric, Ed., New York: Marcel Dekker, 1993, pp. 329-371.

[3] M. Deleu and M. Paquot, “From renewable vegetables resources to microorganisms: New trends in surfactants,” *C. R. Chim*, vol. 7, pp. 641-646, 2004.

[4] A. Singh, J. D. Van Hamme, and O. P. Ward, “Surfactants in microbiology and biotechnology: Part 2. Application aspects,” *Biotechnol. Adv.*, vol. 25, pp. 99-121, 2007.

[5] B. S. Sekhon, “Surfactants: Pharmaceutical and medicinal aspects,” *J. Pharm. Technol. Res. Manage.*, vol. 1, pp. 11-36, 2013.

[6] G. G. Ying, “Fate, behavior and effects of surfactants and their degradation products in the environment,” *Environ. Int.*, vol. 32, pp. 417-431, 2006.

[7] Â. S. Inácio, K. A. Mesquita, M. Baptista, J. Ramalho-Santos, W. L. C. Vas, and O. V. Vieira, “*In Vitro* surfactant structure-toxicity relationships: Implications for surfactant use in sexually transmitted infection prophylaxis and contraception,” *PLoS One*, vol. 6, no. 5, 2011.

[8] F. Hosseini, F. Malekzadeh, N. Amirmozafari, and N. Ghaemi, “Biodegradation of anionic surfactants by isolated bacteria from activated sludge,” *Int. J. Environ. Sci. Tech.*, vol. 4, no. 1, pp. 127-132, 2007.

[9] N. Lourith, and M. Kanlayavattanakul, “Natural surfactants used in cosmetics: Glycolipids,” *Int. J. Cosmetic Sci.*, vol. 31, pp. 255-261, 2009.

[10] K. K. Guatam, and V. K. Tyagi, “Microbial surfactants: A review,” *J. Oleo. Sci.*, vol. 55, pp. 155-166, 2006.

[11] M. S. Yeh, Y. H. Wei, and J. S. Chang, “Enhanced production of surfactin from *Bacillus subtilis* by addition of solid carriers,” *Biotechnol. Progr.*, vol. 21, no. 4, pp. 1329-1334, 2005.

[12] M. Nitschke and S. G. V. A. O. Costa, “Biosurfactants in food industry,” *Trends Food Sci. Tech.*, vol. 18, pp. 252-259, 2007.

[13] K. Muthusamy, S. Gopalakrishnan, T. K. Ravi, and P. Sivachidambaram, “Biosurfactants: Properties, commercial production and application,” *Curr. Sci.*, vol. 94, no. 6, pp. 736-747, 2008.

[14] N. Kosaric, “Biosurfactants in industry,” *Pure. Appl. Chem.*, vol. 64, no. 11, pp. 1731-1737, 1992.

[15] M. Morikawa, Y. Hirata, and T. Imanaka, “A study on the structure function relationship of lipopeptide biosurfactants,” *Biochim. Biophys. Acta*, vol. 1488, no. 3, pp. 211-218, 2000.

[16] A. Aparna, G. Srinikethan, and H. Smitha, “Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B,” *Colloid Surface. B*, vol. 95, pp. 23-29, 2012.

[17] G. S. Kiran, T. A. Thomas, and J. Selvin, “Production of new glycolipid biosurfactant from marine *Nocardiopsis lucentensis* MSA04 in solid state cultivation,” *Colloid Surface. B*, vol. 78, pp. 8-16, 2010.

[18] G. S. Kiran, T. A. Thomas, J. Selvin, B. Sabarathnam, and A. P. Lipton, “Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture,” *Bioresource Technol.*, vol. 101, pp. 2389-2396, 2010.

[19] B. B. Lee, E. S. Chan, P. Ravindra, and T. A. Khan. “Surface tension of viscous biopolymer solutions measured using the du Nouy ring method and the drop weight methods,” *Polym. Bull.*, vol. 69, pp. 471-489, 2012.

[20] A. Khopade, B. Ren, X. Liu, K. Mahadik, L. Zhang, and C. Kokare, “Production and characterization of biosurfactant from marine *Streptomyces* species B3,” *J. Colloid Interf. Sci.*, vol. 367, pp. 311-318, 2012.

[21] W. Ismail, I. S. Al-Rowaihi, A. A. Al-Humam, R. Y. Hamza, A. M. El Nayal, and M. Bououdina, “Characterization of a lipopeptide biosurfactant produced by a crude-oil-emulsifying *Bacillus* sp. I-15,” *Int. Biodeter. Biodegr.*, vol. 84, pp. 168-178, 2012.

[22] S. Joshi, C. Bharucha, and A. J. Desai, “Production of biosurfactant and antifungal compound by fermented food isolate *Bacillus subtilis* 20B,” *Bioresource Technol.*, vol. 99, pp. 4603-4608, 2008.

[23] J. Thaniyawarn, N. Roongsawang, T. Kameyama, M. Haruki, T. Imanaka, M. Morikawa, and S. Kanaya, “Production and characterization of biosurfactants from *Bacillus licheniformis* F2.2,” *Biosci. Biotech. Bioch.*, vol. 67 pp. 1239-1244, 2003.

[24] N. I. A. Haddad, J. Wang, and B. Mu, “Identification of biosurfactant producing strain: *Bacillus subtilis* HOB2,” *Protein Peptide Lett*, vol. 16, pp. 7-13, 2009.

[25] C. Burgos-Díaz, R. Pons, M. J. Espuny, F. J. Aranda, J. A. Teruel, A. Manresa, A. Ortiz, and A. M. Marqués, “Isolation and partial characterization of a biosurfactant mixture produced by *Sphingobacterium* sp. isolated from soil,” *J. Colloid Interf. Sci.*, vol. 361, pp. 195-204, 2011.

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