Production of lipopeptide biosurfactant by *Bacillus subtilis* GY19 and its application as oil-contaminated surface cleaning agent

Sitti Tathong	extsuperscript{a}, Chanokporn Muangchinda	extsuperscript{a,b}, Chayada Kongswan	extsuperscript{b}, Nichakorn Khondee	extsuperscript{c}, Ekawan Luepromchai	extsuperscript{b,d}, Suwat Soonglerdsongpha	extsuperscript{a}, Chalermchai Ruangchainikom	extsuperscript{c}, Onruthai Pinyakong	extsuperscript{b,d,*}

	extsuperscript{a} International Postgraduate Programs in Hazardous Substance and Environmental Management, Graduate School, Chulalongkorn University, Bangkok 10330 Thailand

	extsuperscript{b} Microbial Technology for Marine Pollution Treatment Research Unit, Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330 Thailand

	extsuperscript{c} Department of Natural Resources and Environment, Faculty of Agriculture, Natural Resources and Environment, Naresuan University, Phitsanulok 65000 Thailand

	extsuperscript{d} Research Program on Remediation Technologies for Petroleum Contamination, Center of Excellence on Hazardous Substance Management (HSM), Bangkok 10330 Thailand

	extsuperscript{e} Environmental Research and Management Department, PTT Research and Technology Institute, Ayutthaya 13170 Thailand

	extsuperscript{*}Corresponding author, e-mail: onruthai@gmail.com

**ABSTRACT:** The present study investigated the effect of nutrient composition and pH on the lipopeptide production by *Bacillus subtilis* GY19. The maximum lipopeptide production (2.2 g/l) was achieved when the strain was grown in productive medium containing glycerol (4 g/l) and palm oil (0.75%, v/v) as substrates, sodium nitrate (0.5%, w/v) as nitrogen source, and glucose (1 g/l) and beef extract (0.5 g/l) as co-substrates with pH 7.5. In addition, the lipopeptide of *B. subtilis* GY19 could be applied for removal of slideway oil covered on metallic surface. Taguchi method was employed to evaluate the factors affecting the cleaning process. The results indicated that the presence of high levels of crude lipopeptide concentration positively affected surface washing efficacy. Further removal of slideway oil from the washing water could be achieved by the addition of immobilized oil-degrading bacterium, *Acinetobacter* sp. R2. The presence of lipopeptide increased the removal efficiency of slideway oil from 70% to 82% and did not show toxic effect on bacterial cells. This study shows promising ability of the lipopeptide from *B. subtilis* GY19 as a cleaning agent for oil-contaminated surface. In addition, it could subsequently enhance biodegradation of residual oil in the washing water.

**KEYWORDS:** biosurfactant, surface washing, oil removal, lipopeptide, *Bacillus*

**INTRODUCTION**

Biosurfactants are surface-active substances synthesized by bacteria, yeast, and filamentous fungi. They are amphiphilic molecules with both hydrophilic and hydrophobic fragments preferentially partitioned at the interfaces between phases, which have different degrees of polarity and hydrogen bonding such as oil-water or air-water interfaces [1]. The biosurfactants produced by bacteria are classified into 4 types based on their chemical composition: glycolipids, lipopeptides, phospholipids, and polymeric surfactants [2]. Lipopeptides are the most known types of biosurfactants which are normally produced by members of *Bacillus* species and possess good surface-active characteristics [3].

Biosurfactants have increasingly attracted attention due to their safe and environmentally sustainable properties [4]. Biosurfactants have shown numerous environmental applications such as enhancing oil recovery and remediation of oil-contaminated environments [5, 6]. Based on the properties of biosurfactants, their applications as alternative petroleum oil-contaminated surface cleaning agents are interesting [7, 8]. The biosurfactants are expected to enhance oil removal from surface and will not cause further hazardous waste as using chemical solvent. In addition, biosurfactants might also facilitate biodegradation of residual petroleum oil in subsequent treatment system. However, the use of biosurfactants is limited by the low biosurfactant yield. The optimization of production medium and conditions for maximizing the yield of biosurfactant could contribute to expanding the biosurfactant applications [9]. A variety of factors was reported to influence the biosurfactant production such as carbon and nitrogen sources, pH, temperature, time of cultivation, and agitation speed [10–12].

*Bacillus subtilis* GY19 was previously isolated from soil samples in Thailand. This strain was immobilized on chitosan to produce biosurfactant, and it has been demonstrated to be an efficient lipopeptide biosurfactant producer using waste glycerol and palm oil as
substrates. The components of lipopeptide from GY19 were previously investigated, and the major lipopeptide in this bacterium is the surfactin isoform [13]. However, the scale-up production of biosurfactants by immobilized cells is quite complicated and difficult. In the present study, the production of lipopeptides by free cells of *B. subtilis* GY19 was optimized to be more practical in the future scale-up production. The objectives of this study were as follows: (1) to investigate the effect of nutrient composition and pH on the lipopeptide production from *B. subtilis* GY19; (2) to apply the produced lipopeptide for washing an oil-contaminated surface and to investigate the effects of heating temperature, heating time, shaking time, shaking speed, and crude lipopeptide concentration on washing efficiency; and (3) to determine the influence of lipopeptide and several chemosynthetic surfactants on oil biodegradation.

**MATERIALS AND METHODS**

**Lipopeptide-producing bacteria and culture condition**

The lipopeptide-producing strain *Bacillus subtilis* GY19 previously isolated from soil samples in Thailand was used in the present study [13]. The productive medium used for biosurfactant production was modified from Nawawi et al. [14], which contains (g/l): glucose (1.0); beef extract (0.5); KH₂PO₄ (3.3); K₂HPO₄ (0.14); NaNO₃ (0.2); NH₄NO₃ (3.3); CaCl₂ (0.04); NaCl (0.04); MgSO₄·7H₂O (0.3); FeSO₄·7H₂O (0.1); and waste glycerol (4.0), and the initial pH of the medium was 6.3. Waste glycerol was obtained from Thai Oleochemicals Co., Ltd. (Thailand). It contained (g/l): glycerol (190); sodium (17.5); potassium (43.8); sulfate (22.7); phosphate (0.2); total nitrogen (0.1); and COD (1069) [13]. *B. subtilis* GY19 was cultivated on a rotary shaker at 200 rpm, room temperature for 5 days as mentioned in our previous study [13].

**Effects of nitrogen sources, co-substrates, pH, and lipophilic substrates on lipopeptide production**

Inoculum of GY19 was prepared in 100 ml of Luria-Bertani (LB) broth [15], and the culture was incubated at room temperature and shaken at 200 rpm for 24 h. Bacterial cells were harvested by centrifugation at 8000 rpm at 4 °C for 20 min, and the bacterial pellet was suspended in 0.85% (w/v) NaCl solution. The optical density at 600 nm was adjusted to 1.0. The inoculum (3% v/v) was added to the productive medium and incubated as described above. The effects of media components and pH on optimization of lipopeptide production were examined. In this study, glycerol (4 g/l) was used as a hydrophilic substrate. The effect of nitrogen source was studied by comparing (NH₄)₂SO₄, NaNO₃, and NH₄NO₃ as sole nitrogen source. The nitrogen sources were added to the culture medium at 0.1, 0.35, and 0.5% (w/v). The effect of co-substrate was evaluated by supplementing glucose and/or beef extract to the medium. The pH of the medium was varied from 6.5 to 7.5. The influence of type and concentration of lipophilic substrates including commercial soybean oil, rice bran oil, and palm oil for biosurfactant production was investigated at 0.25, 0.5, and 0.75% (v/v). After incubation, the total amount of biomass was measured in terms of dry cell weight. Biosurfactant production was analyzed in terms of surface tension reduction and biosurfactant yield. Surface tension activity of cell free supernatant was measured by using Tensiometer (Dataphysics, DCAT 11EC, Germany). The biosurfactant yield was calculated from the amount of crude lipopeptide per liter of the production medium.

**Extraction of crude lipopeptide**

To extract the produced lipopeptide from culture medium, the bacterial cells were separated by centrifugation at 8000 rpm for 15 min. The residual vegetable oil in supernatant was removed by hexane extraction. The obtained supernatant was then acidified to pH 2 with 6 M HCl and incubated overnight at 4 °C. Supernatant containing biosurfactant was extracted 3 times with equal volume of chloroform-methanol (2:1). The lower solvent phase was collected and evaporated by a rotary evaporator to recover the crude lipopeptide. The amount of crude lipopeptide was measured by using an analytical balance. In this study, quantification of biosurfactant after solvent extraction from culture supernatant was used to preliminarily determine the biosurfactant concentration. The lipopeptide purification process was not conducted since the crude lipopeptide extract was used for preparing washing solutions in the following experiment.

**Surface washing with produced lipopeptide and optimization of washing conditions**

The application of the produced lipopeptide for cleaning a contaminated surface with a layer of oil was evaluated. The contamination of slideway oil (PTT slideway oil 68; PTT Public Co. Ltd., Thailand) on hard surface was carried out by blotting 0.02 g of slideway oil on the 5 × 5 cm² stainless steel for 24 h and dried in oven at 70 °C. Slideway oil is a lubricating oil usually

### Table 1 Controllable factors and their levels in the Taguchi method.

| Factor                  | Unit | Level |
|-------------------------|------|-------|
| Lipopeptide concentration | g/l  | 0 0.7 1.4 5.6 |
| Heating temperature     | °C   | 80 100 120 140 |
| Heating time            | min  | 60 90 120 150 |
| Shaking time            | min  | 60 80 100 120 |
| Shaking speed           | rpm  | 75 100 125 150 |
used for machine tool slides and tables in factories. Posteriorly, the contaminated metal was immersed in 300 ml of crude lipopeptide solution. To assess the effect of heating temperature, heating time, shaking time, shaking speed, and crude lipopeptide concentration on oil-degraded surface cleaning efficiency, the washing condition was designed by using Taguchi method. The parameters were selected based on the cleaning operation conditions in factories. For example, machines in petroleum refining were operated under high temperature ranging from 200 to 600 °C [16]. Although oil contaminated machines were cleaned after cooling down, the temperature would be higher than 100 °C. In addition, the high temperature can affect the oil transport such as reducing viscosity and decreasing the persistence of the stranded oil.

Table 1 shows the ranges and levels of different independent variables of cleaning conditions, which were designed for Taguchi method. After washing, the amount of removed oil was determined by Horiba OCMA-310 oil content analyzer (Horiba, Japan). The removal percentage of oil from stainless steel surface was calculated for each experiment by following equation (Eq. (1)):

\[
\text{Oil removal efficiency}\% = \frac{M_i - M_r}{M_i} \times 100 \quad (1)
\]

where \(M_i\) and \(M_r\) are initial and residual oil on stainless steel (mg), respectively.

The Taguchi method utilizes an orthogonal array (OA) for experimental design and applies the signal-to-noise ratio (S/N) for quality evaluation [17]. As the purpose of this study was to remove maximum oil from stainless steel surface, the S/N ratio with the larger-the-better characteristic was needed. Therefore, the S/N calculation conforming to the larger-the-better was determined by applying following equation (Eq. (2)):

\[
S/N = -10\log\left(1/(n \sum (1/PRE))\right) \quad (2)
\]

where \(n\) is the number of experiments under similar experimental condition, and PRE is the results of measurements.

**Oil biodegradation in the presence of produced lipopeptide**

To compare the enhancing oil removal efficiencies between biosurfactants and synthetic surfactants, crude lipopeptide, tween 80, sodium dodecyl sulfate (SDS), and cetyltrimethylammonium bromide (CTAB) were supplemented in media containing slideway oil- and immobilized oil-degrading bacteria. The petroleum oil-degrading strain Acinetobacter sp. R2 (MSCU 0467) obtained from the culture collection of the Department of Microbiology, Faculty of Science, Chulalongkorn University, Thailand was used in this experiment. The bacterium was immobilized on plastic pellets (2H GmbH, Germany) to increase cell density and activity. Inoculum of R2 was added to 50 ml of 2-fold-diluted LB medium supplemented with 20 µl of slideway oil and 5 g of sterilized plastic pellets and incubated for 24 h. The number of R2 cells immobilized on the carrier material were approximately \(10^9\) CFU/g. The degradation experiments were performed by adding 5 g of immobilized cells into 50 ml carbon free mineral medium (CFMM) [18] containing 300 mg/l of slideway oil and shaken at 200 rpm at room temperature for 5 days. A crude lipopeptide and synthetic surfactants were added to the samples at 1 × CMC and 5 × CMC. The selected surfactant concentrations allowed the micelle formation, which could promote petroleum solubilization in the system. After incubation, the residual oil was quantified using thin-layer chromatography with flame ionization detection (TLC-FID) (Iatron Labs, Tokyo, Japan) as described by Nopcharoenkul et al [19]. The study in the absence of biosurfactant was carried out as control. All experiments were carried out in triplicate. The inhibitory effect of the surfactant towards bacterial growth was studied using viable plate count technique on LB agar.

### RESULTS AND DISCUSSION

**Influence of nutrient composition and pH on the production of lipopeptide**

The production of biosurfactant in microorganisms is strongly influenced by medium composition and other physical parameters, hence optimization of culture conditions can be used to maximize the biosurfactant yield [20]. The effects of varying nitrogen sources, co-substrates, pH, and lipophilic substrates on lipopeptide production by *Bacillus subtilis* GY19 are indicated in Table 2. Nitrogen source is one of the important factors to influence the biosurfactant production [21]. Table 2 shows that sodium nitrate at 0.5% (w/v) was the best in producing the lipopeptide with a yield of 1.7 g/l (and surface tension 28.4 mN/m); therefore, 0.5% (w/v) sodium nitrate was noted as the suitable nitrogen source and used for further experiments. Several studies have shown the influence of nitrogen source on the production of biosurfactant. Sodium nitrate is frequently used and found to give the high biosurfactant production yield [22, 23].

In the next optimization, effect of co-substrate on the production of lipopeptide was studied. Data in Table 2 revealed that the highest lipopeptide production was achieved when adding both glucose and beef extract as co-substrates. From our data, it was noted that glucose (1 g/l) and beef extract (0.5 g/l) were the potential co-substrates for the lipopeptide production. These conditions were chosen for further experiments. Raza et al [24] demonstrated that the production of rhamnolipids by *Pseudomonas putida* 33 using waste frying oil as carbon source increased when adding glu-
Table 2  Effects of nitrogen, co-substrates, pH, and lipophilic substrates on lipopeptide production by *Bacillus subtilis* GY19.

| Experiment                        | Surface tension (mN/m) | Dried cell weight (g/l) | Crude lipopeptide (g/l) |
|-----------------------------------|------------------------|-------------------------|-------------------------|
| **Effect of nitrogen**            |                        |                         |                         |
| (NH₄)₂SO₄ 0.10%                   | 39.0±3.0               | 2.2                     | 0.6                     |
|                                  | 39.0±3.0               | 2.2                     | 1.6                     |
|                                  | 38.0±1.0               | 1.9                     | 1.6                     |
| NaNO₃ 0.10%                       | 29.7±0.4               | 2.3                     | 0.9                     |
|                                  | 28.8±0.1               | 2.3                     | 1.2                     |
|                                  | 28.4±0.4               | 2.2                     | 1.7                     |
| NH₄NO₃ 0.10%                      | 29.3±0.3               | 1.9                     | 0.7                     |
|                                  | 30.8±0.2               | 1.9                     | 0.9                     |
|                                  | 29.3±0.3               | 1.8                     | 1.3                     |
| **Effect of co-substrate**        |                        |                         |                         |
| Both glucose and beef extract    | 28.4±0.4               | 2.2                     | 1.7                     |
| Only glucose                     | 29.9±0.4               | 1.2                     | 1.1                     |
| Only beef extract                | 34.3±0.9               | 0.8                     | 0.5                     |
| None                             | 34.3±0.6               | 1.0                     | 0.7                     |
| **Effect of pH medium**          |                        |                         |                         |
| pH 6.5                           | 28.6±0.6               | 2.1                     | 1.6                     |
| pH 7.0                           | 28.5±0.5               | 2.2                     | 1.7                     |
| pH 7.5                           | 28.2±0.4               | 2.0                     | 1.8                     |
| **Effect of lipophilic substrate**|                        |                         |                         |
| Soybean oil 0.25%                | 34.5±0.6               | 1.8                     | ND                      |
|                                  | 34.4±0.2               | 1.7                     | ND                      |
|                                  | 30.5±1.5               | 1.9                     | 1.5                     |
| Palm oil 0.25%                   | 33.4±0.2               | 1.9                     | ND                      |
|                                  | 30.2±0.9               | 1.8                     | ND                      |
|                                  | 29.7±0.5               | 2.0                     | 2.2                     |
| Rice bran oil 0.25%              | 35.4±0.2               | 1.7                     | ND                      |
|                                  | 31.6±0.5               | 1.8                     | ND                      |
|                                  | 29.3±0.4               | 2.0                     | 1.9                     |

ND, not determined.

cose as co-substrate. In addition, Kiran et al [25] found that the beef extract showed significant increase in the production of glycolipid by *Nocardiopsis lucentensis* MSA04.

Change in pH is one of the environmental factors to influence the biosurfactant production [26]. The effects of pH on the production of lipopeptide by *B. subtilis* GY19 were investigated by varying the pH of the culture medium from 6.5 to 7.5 (Table 2). In the present study, the production of lipopeptide was not different under different pH values. The maximum yield recorded at pH 7.5 was 1.8 g/l. Hence, the optimum pH was noted as 7.5 and used for further experiments.

To enhance lipopeptide production, soybean, rice bran, and palm oil were used as lipophilic substrates for lipopeptide production in this study in the optimal nutrient and pH obtained above. Different vegetable oils enhanced lipopeptide production differentially; maximum production occurred in the presence of palm oil followed by rice bran oil and soybean oil, respectively (Table 2). The use of vegetable oil as the lipophilic substrate was found to enhance the production of a biosurfactant. Qazi et al [27] observed that the addition of 2% olive oil could enhance biosurfactant production by *Pseudomonas putida* SOL-10.

In this study, the maximum lipopeptide yield at 2.2 g/l and surface tension of 29.7±0.5 mN/m were obtained by using productive medium consisting of glycerol (4 g/l) as hydrophilic substrate, palm oil (0.75%, v/v) as lipophilic substrate, sodium nitrate (0.5%, w/v) as nitrogen source, glucose (1 g/l) and beef extract (0.5 g/l) as co-substrates with pH 7.5. The yield of lipopeptides obtained in the present study was found either higher than or comparable with that from other reports at shake flask level. For example, Vigneshwaran et al [28] showed that the highest lipopeptide yield of 1.29 g/l was achieved under the optimized conditions with the addition of 0.9% (v/v) used engine oil and 0.53% (w/v) potassium nitrate as carbon source and nitrogen source, respectively, at pH 7.1. Another result was observed by Sharma et al [29] who optimized the culture conditions for maximum lipopeptide yield by *Bacillus amyloliquefaciens* SAS-1 and *B. subtilis* BR-15 and found that lipopeptide yield was increased from 1.13 to 2.08 g/l for strain SAS-1 and 1.72 to 2.40 g/l for strain BR-15. Nevertheless, lipopeptide biosurfactants by GY19 may be produced with the higher yield. Other process parameters such as temperature, inoculum size, and incubation time.
The application of crude lipopeptide for cleaning oil-contaminated metallic surface was evaluated. To minimize the number of tests required and maximize the effectiveness, some efficient experimental designs, i.e., orthogonal design and Taguchi method can be applied to address multifactor experiments, and the screening of optimum levels by using an orthogonal design table can be used [32]. In this study, effects of heating temperature, heating time, shaking time, shaking speed, and crude lipopeptide concentration on slideway-contaminated surface washing efficiency were studied by Taguchi method, and the results of the 16 experiments are summarized in Table 3. Taguchi method was particularly designed by using a numerical value called signal-to-noise (S/N) ratio to evaluate all the experiments. Moreover, this ratio is very much helpful for estimation of best combination of factors [33]. As a result, lipopeptide concentration had the highest S/N ratio, which indicated the greatest effect on oil cleaning efficiency followed by shaking speed, shaking time, heating temperature, and heating time, respectively (Fig. 1). The optimum level of each factor was determined from the highest value of S/N ratio. The optimum conditions to achieve the maximum oil removal were found to be lipopeptide concentration of 5.6 g/l, shaking speed of 150 rpm, shaking time of 100 min, heating temperature of 120°C, and heating time of 150 min (Table 4). Effect size helps understand the magnitude of differences found, whereas statistical significance examines whether the findings are likely to be due to chance [34]. Thus, concentration of crude lipopeptide was the crucial factor. The optimal concentration of crude lipopeptide was higher than its critical micelle concentration (CMC) of 1.4 g/l [35]. The high oil cleaning efficiency at above CMC of lipopeptide demonstrated that the solubilization of oil into lipopeptide micelle was the main cleaning mechanism. Micelles are important in cleaning because they can solubilize insoluble oils by incorporating and trapping them within the micellar structure [36]. The mechanisms were expected to involve (1) adsorption of micelles on stainless steel surface; (2) solubilization of oils into micelles; and (3) desorption of oil-containing micelles. Similarly, Zheng et al [37] observed that oil recovery from oil sludge by a biosurfactant formula was higher with the increase of biosurfactant concentration. This study showed that biosurfactants displayed oil removal properties from the solid surfaces and was in accordance with the studies summarized by previous reviews [7,8]. For instance, Silva et al [38] reported that the biosurfactant produced by Pseudomonas cepacia CCT6659 could be applied for cleaning beaker walls contaminated with an oil layer.

Effects of produced lipopeptide and chemical surfactant addition on oil removal

To degrade slideway oil in the washing water, crude lipopeptide from B. subtilis GY19 and other chemical surfactants were added to an artificial wastewater treatment system containing the immobilized oil-degrading Acinetobacter sp. R2 cells. The oil removal efficiencies in the presence of crude lipopeptide from GY19 compared with chemical surfactants (tween 80, SDS, and CTAB) are shown in Fig. 2a. The results demonstrated that the addition of lipopeptide at 1 × CMC (1.4 g/l) and 5 × CMC (7.0 g/l) increased the slideway oil removal percentage from 70.04% in the treatment without surfactant to 77.29 and 82.10%, respectively. The addition of the tween 80 and SDS at 1 × CMC also increased the slideway oil removal efficiencies; however, the removal efficiencies decreased with increasing concentrations of these synthetic surfactants. In the case of CTAB, the oil removal performance obtained using this surfactant was low.

Furthermore, the effect of surfactant solution on the survival of strain R2 was investigated. As shown in Fig. 2b, tween 80 and SDS at 1 × CMC had no negative effect on immobilized R2 cells. The number of bacteria on the plastic pellets were not different from a control treatment (no addition of surfactant). When the concentration of tween 80 and SDS increased to 5 × CMC, the number of R2 cells decreased resulting in a decrease in the removal efficiency of immobilized R2 cells. For CTAB, it showed negative effect on immobilized R2 cells at both concentrations (1 × CMC and 5 × CMC). Bucci et al [39] suggested that CTAB has good properties for the antimicrobial activity. Moreover, CTAB is a known chemical used for microbial cell lysis [40]. In the treatment containing the biosurfactant, the number of R2 cells at both lipopeptide con-

Fig. 1 Main effect plots (S/N ratio) for oil removal efficiency. Signal-to-Noise Ratio: The larger-The better.
Table 3  Taguchi design for optimization of cleaning conditions, oil cleaning efficiency, and S/N ratio.

| Run | Independent variable | Dependent variable | S/N ratio |
|-----|----------------------|--------------------|----------|
| No. | Lipopeptide concentration (g/l) | Heating temperature (°C) | Heating time (min) | Shaking time (min) | Shaking speed (rpm) | Oil removal efficiency (%) |
| 1   | 0.0                  | 80                 | 60        | 60              | 75              | 0.11                | -19.1721                  |
| 2   | 0.0                  | 100                | 90        | 80              | 100             | 0.68                | -3.3498                   |
| 3   | 0.0                  | 120                | 120       | 100             | 125             | 9.95                | 19.9565                   |
| 4   | 0.0                  | 140                | 150       | 120             | 150             | 11.10               | 20.9065                   |
| 5   | 0.7                  | 80                 | 90        | 120             | 125             | 10.34               | 20.2904                   |
| 6   | 0.7                  | 100                | 60        | 100             | 150             | 8.75                | 18.8402                   |
| 7   | 0.7                  | 120                | 150       | 80              | 75              | 5.46                | 14.7439                   |
| 8   | 0.7                  | 140                | 120       | 60              | 100             | 7.10                | 17.0252                   |
| 9   | 1.4                  | 80                 | 120       | 80              | 150             | 18.00               | 25.1055                   |
| 10  | 1.4                  | 100                | 60        | 120             | 125             | 26.43               | 28.4419                   |
| 11  | 1.4                  | 120                | 150       | 120             | 100             | 42.48               | 32.5637                   |
| 12  | 1.4                  | 140                | 90        | 100             | 75              | 21.56               | 26.6730                   |
| 13  | 5.6                  | 80                 | 150       | 100             | 100             | 100.00              | 40.0000                   |
| 14  | 5.6                  | 100                | 120       | 120             | 75              | 86.85               | 38.7754                   |
| 15  | 5.6                  | 120                | 90        | 60              | 150             | 100.00              | 40.0000                   |
| 16  | 5.6                  | 140                | 60        | 80              | 125             | 91.17               | 39.1970                   |

Fig. 2 Effects of addition of surfactant solution on (a) slideway oil removal by immobilized *Acinetobacter* sp. R2 and (b) the number of *Acinetobacter* sp. R2 on carrier materials. The critical micellar concentration (CMC) of tween 80, SDS, and CTAB in water is estimated to be around 0.01, 8 and, 0.9 mM, respectively.

Table 4 The optimum factors for slideway oil cleaning from stainless steel surface.

| Factor                | Unit | Level description | Effect size |
|-----------------------|------|-------------------|-------------|
| Lipopeptide concentration | g/l  | (5.6)4            | 16.9933     |
| Heating temperature   | °C   | (120)3            | 4.3162      |
| Heating time          | min  | (150)4            | 3.5232      |
| Shaking time          | min  | (100)3            | 4.4716      |
| Shaking speed         | rpm  | (150)4            | 5.6342      |

Expected $S/N = 57.4384$. Signal-to-Noise Ratio: The larger-The better.

The oil removal efficiencies in the presence of crude lipopeptide from GY19 compared with tween 80 and SDS at 1 × CMC were not significantly different and did not affect the survival of strain R2. However, when the surfactant concentration was higher than the CMC which was recommended for enhancing hydrocarbon degradation, it was found that tween 80 and SDS had a negative effect on the R2 cells. On the other hand, the highest oil removal was achieved when adding crude lipopeptide from GY19 at 5 × CMC without any negative effect on the R2 cells. These results demonstrated that lipopeptide from *B. subtilis* GY19 could be applied to promote biodegradation of oil in a subsequent wastewater treatment system.

**CONCLUSION**

In this study, the effects of culture media components and pH on the lipopeptide production by *Bacillus subtilis* GY19 were investigated. The maximum lipopeptide yield of 2.2 g/l was obtained from the optimized production medium, which contained 4 g/l glycerol, 0.75% (v/v) palm oil, 0.5% (w/v) sodium nitrate, 1 g/l glucose, and 0.5 g/l beef extract at pH 7.5.
The produced lipopeptide showed the effective action in the cleaning of slideway oil-contaminated metallic surface. The concentration of crude lipopeptide was a variable that most influenced oil washing efficiency. Furthermore, the presence of the produced lipopeptide caused a positive effect on slideway oil degradation by the immobilized R2 cells and did not exhibit any toxic effect to bacterial cells. These results indicated that B. subtilis GY19 lipopeptides could be used as an alternative oil-contaminated surface cleaning agent and could promote biodegradation of oil in a subsequent wastewater treatment system.

Acknowledgements: This study was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the PTT Research and Technology. ST was supported by the Center of Excellence on Hazardous Substance Management (HSM) for Doctoral Scholarship.

REFERENCES

1. Santos DKE, Rufino RD, Luna JM, Santos VA, Sarubbo LA (2016) Biosurfactants: multifunctional biomolecules of the 21st century. Int J Mol Sci 17, ID 401.
2. Varjani SJ, Upasani VN (2017) Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. Bioresour Technol 232, 389–397.
3. Nimrat S, Lookchan S, Boonthai T, Vuthiphandchai V (2020) Isolation, optimisation and gasoline biodegradation by lipopeptide-producing Bacillus subtilis SE1. ScienceAsia 46, 195–205.
4. Usman MM, Dadraria A, Lim KT, Mahmud AF, Ismail S (2016) Application of biosurfactants in environmental biotechnology: remediation of oil and heavy metal. AIM Bioeng 3, 289–304.
5. Souza EC, Penna TCV, Oliveira RPS (2014) Biosurfactant-enhanced hydrocarbon bioremediation: an overview. Int Biodeter Biodegradation 89, 88–94.
6. Shekhar S, Sundaramanickam A, Balasubramanian T (2015) Biosurfactant producing microbes and their potential applications: a review. Crit Rev Environ Sci Technol 45, 1522–1554.
7. Silva RFS, Almeida DG, Rufino RD, Luna JM, Santos VA, Sarubbo LA (2014) Applications of biosurfactants in the petroleum industry and the remediation of oil spills. Int J Mol Sci 15, 12523–12542.
8. Fenibo EO, Ijoma GN, Selvarajan R, Chikere CB (2019) Microbial surfactants: The next generation multifunctional biomolecules for applications in the petroleum industry and its associated environmental remediation. Microorganisms 7, ID 581.
9. Najafi AR, Rahimpoura MR, Jahanmiria AH, Roostaazad R, Arabianb D, G Hobadi Z (2010) Enhancing biosurfactant production from an indigenous strain of Bacillus mycoides by optimizing the growth conditions using a response surface methodology. Chem Eng J 163, 188–194.
10. Ozdal M, G urkok S, Ozdal OG (2017) Optimization of rhamnolipid production by Pseudomonas aeruginosa OG1 using waste frying oil and chicken feather peptone. J Biotech 7, ID 117.
11. Vigneshwaran C, Sivasubramanian V, Vasantharaj K, Krishnanand N, Jerold M (2018) Potential of Brevibacillus sp. AVN 13 isolated from crude oil contaminated soil for biosurfactant production and its optimization studies. J Environ Chem Eng 6, 4347–4356.
12. Santos DKE, Brandão YB, Rufino RD, Luna JM, Salgueiro AA, Santos VA, Sarubbo LA (2014) Optimization of cultural conditions for biosurfactant production from Candida lipolytica. Biocatal Agric Biotechnol 3, 48–57.
13. Khondee N, Tathong S, Pinyakong O, Müller R, Soonglersongpha S, Ruangchainikom C, Tongcumpou C, Luempromchai E (2015) Lipopeptide biosurfactant production by chitosan-immobilized Bacillus sp. GY19 and their recovery by foam fractionation. Biochem Eng J 93, 47–54.
14. Nawawi WMFW, Jamal P, Alam MZ (2010) Utilization of sludge palm oil as a novel substrate for biosurfactant production. Bioresour Technol 101, 9241–9247.
15. Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogetic Escherichia coli. J Bacteriol 62, 293–300.
16. Al-Jamimi HA, BinMakahesh GM, Deb K, Saleh TA (2021) Multiobjective optimization and analysis of petroleum refinery catalytic processes: A review. Fuel 288, ID 119678.
17. Abbasi F, Yaraki MT, Farrokhnia A, Bamdad M (2020) Keratin nanoparticles obtained from human hair for removal of crystal violet from aqueous solution: Optimized by Taguchi method. Int J Biol Macromol 143, 492–500.
18. Wongwongsee W, Charanpat P, Pinyakong O (2013) Abilities and genes for PAH biodegradation of bacteria isolated from mangrove sediments from the central of Thailand. Mar Pollut Bull 74, 95–104.
19. Nophcharoenkul W, Netsakulnee P, Pinyakong O (2013) Diesel oil removal by immobilized Pseudoxanthomonas sp. RN402. Biodegradation 24, 387–397.
20. Jahan R, Bodratti AM, Tsianou M, Alexandridis P (2020) Biosurfactants, natural alternatives to synthetic surfactants: Physicochemical properties and applications. Adv Colloid Interface Sci 275, 102061.
21. Nurfarahin AH, Mohamed MH, Phang LY (2018) Culture medium development for microbial-derived surfactants production: An overview. Molecule 23, ID 1049.
22. Onwosi CO, Odibo FJC (2012) Effects of carbon and nitrogen sources on rhamnolipid biosurfactant production by Pseudomonas nitroreducens isolated from soil. World J Microbiol Biotechnol 28, 937–942.
23. Agarry SE, Salam KK, Arinkoola A, Aremu MO (2015) Biosurfactant production by indigenous Pseudomonas and Bacillus species isolated from auto-mechanic soil environment toward microbial enhanced oil recovery. Eur J Eng Technol 3, 27–39.
24. Raza ZA, Khan MS, Khalid ZM (2007) Evaluation of distant carbon sources in biosurfactant production by a gamma ray-induced Pseudomonas putida mutant. Process Biochem 42, 686–692.
25. Kiran GS, Thoma, TA, Selvin J (2010) Production of a new glycolipid biosurfactant from marine Nocardiopsis lucentensis MSA04 in solid-state cultivation. Coll Surf B Biointer 78, 8–16.
26. Fakruddin M (2012) Biosurfactant: production and application. J Pet Environ Biotechnol 3, ID 4.
27. Qazi MA, Malik ZA, Qureshi GD, Hameed A, Ahmed S (2013) Yeast extract as the most preferable substrate for optimized biosurfactant production by rhlB gene positive Pseudomonas putida SOL-10 isolate. *J Bioremed Biodeg* 4, ID 204.

28. Vigneshwaran C, Vasantharaj K, Krishnanand N, Sivasubramanian V (2021) Production optimization, purification and characterization of lipopeptide biosurfactant obtained from *Brevibacillus* sp. AVN13. *J Environ Chem Eng* 9, ID 104867.

29. Sharma R, Singh J, Verma N (2020) Statistical optimization and comparative study of lipopeptides produced by *Bacillus amyloliquefaciens* SAS-1 and *Bacillus subtilis* BR-15. *Biocatal Agric Biotechnol* 25, ID 101575.

30. Mouafi FE, Elsouda MMA, Moharamb ME (2016) Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. *Biotechnol Rep* 9, 31–37.

31. Sharma R, Singh J, Verma N (2018) Optimization of rhamnolipid production from *Pseudomonas aeruginosa* PBS towards application for microbial enhanced oil recovery. *3 Biotech* 8, 20–26.

32. Deng LY (2000) Orthogonal arrays: theory and applications. *Technometrics* 42, 440–440.

33. Chentharamarakan A, Parambayil N, Miziriy N, Soumya P, Lakshmi MK, Ramgopal A, Dileep P, Nambisan P (2017) Optimization of laccase production from *Marasmiellus palmivorus* LA1 by Taguchi method of design of experiments. *BMC Biotechnol* 17, ID 12.

34. Sullivan GM, Feinn R (2012) Using effect size—or why the *P* value is not enough. *J Grad Med Educ* 4, 279–282.

35. Rongsayamanont W, Soonglersongpha S, Khondee N, Pinyakong O, Tongrumpou C, Sabatini AD, Luepromchai E (2017) Formulation of crude oil spill dispersants based on the HLD concept and using a lipopeptide biosurfactant. *J Hazard Mater* 334, 168–177.

36. Rhein L (2007) Surfactant action on skin and hair: Cleansing and skin reactivity mechanisms. In: Johansson I, Somasundaran P (eds) *Handbook for Cleaning/Decontamination of Surfaces*, Elsevier Science, Elsevier, pp. 305–369.

37. Zheng C, Wang M, Wang Y, Huang Z (2012) Optimization of biosurfactant-mediated oil extraction from oil sludge. *Bioresour Technol* 110, 338–342.

38. Silva EJ, Rocha SNM, Rufino RD, Luna JM, Silva RO, Sarubbo LA (2014) Characterization of a biosurfactant produced by *Pseudomonas cepacia* CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Collo Surf B Biointer* 117, 36–41.

39. Bucci AR, Marcelino L, Mendes RK, Etchegaray A (2018) The antimicrobial and antiadhesion activities of micellar solutions of surfactin, CTAB and CPCl with terpinen-4-ol: applications to control oral pathogens. *World J Microbiol Biotechnol* 34, ID 86.

40. Simmon KE, Steadman DD, Dukin S, Baldwin A, Jeffrey WH, Sheridan P, Horton R, Shields MS (2004) Autoclave method for rapid preparation of bacterial PCR-template DNA. *J Microbiol Methods* 56, 143–149.