

Isolation and Characterization of Biosurfactant from *Bacillus atrophaeus* DYL-130

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The objective of this study was investigate the characteristic of biosurfactant produced from the isolated strain. The strain was isolated from soli samples of Duck-Yu Mountain and it was identified as *Bacillus atrophaeus* DYL-130 by 16S rDNA and *gyrA* gene nucleotide sequence analysis. The surface tension of culture filtrate of *Bacillus atrophaeus* DYL-130 decreased to 28 mN/m and its biosurfactant concentration was determined by diluting the culture filtrate until the critical micelle concentration (CMC). The emulsifying activity and stability of crude biosurfactant was measured by using water-immiscible hydrocarbons and oils as substrate. The biosurfactant was purified by affinity chromatography and the surface activity of purified biosurfactant was measured by drop-collapsing method and it could be effectively emulsify toluene.

Key words – Biosurfactant, drop-collapsing method, Affinity chromatography, surface-activity.

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties that divide preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. These properties render surfactants capable of reducing surface and interfacial tension and forming microemulsion where hydrocarbons can solubilize in water or where water solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming, and dispersing traits, which makes surfactants some of the most versatile process chemicals[10,11].

Biosurfactants have several advantages over the chemical surfactants, such as low toxicity, high biodegradability [21] and high surface activity, and are suitable for applications in a variety of industries[2,18]. Although all surfactants to be used until now derived from petroleum, however, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation, and their potential applications in the environmental protection, crude oil recovery, health care, and food-processing industries. Biosurfactants are usually complex lipids, with chemically complicated structures more than synthetic surfactants. There are six classes of biosurfactants ; glyco-

lipids, lipopeptides or lipoproteins, neutral lipids, phospholipids, substituted fatty acids, and lipopolysaccharides[6,7, 8,9,16,19]. Production of an effective lipopeptide type biosurfactant, surfactin, was firstly reported from *Bacillus subtilis*[1].

Here, we isolated a strain from Duck-Yu Mountain and named to *Bacillus atrophaeus* DYL-130. The strain produced novel biosurfactant and it has very strong emulsion activity for various hydrocarbons. We isolated the biosurfactant and measured the surface activity of the sample using drop-collapsing method. Also, we observed that the isolated biosurfactant is powerful emulsifier for toluene.

Materials and Methods

Isolation and cultivation of strain DYL-130.

Bacterial producing oil-degrading biosurfactant was isolated in the soil sample collected from Duck-Yu Mountain. The base mineral medium contains with following composition : (NH₄)₂SO₄ 5 g, K₂HPO₄ 2 g, MgSO₄ · 7H₂O 0.2 g, KH₂PO₄ 1 g, CaCl₂ 10 mg, FeSO₄ · 7H₂O 10 mg, NaCl 30 g, yeast extract 0.2 g and trace element solution 2 ml (MoO₃ 1 mg/l, ZnSO₄ · 7H₂O 7 mg/l, CuSO₄ · 5H₂O 0.5 mg/l, H₃BO₃ 1 mg/l, CoCl₂ · 6H₂O 6 mg/l, NiSO₄ · 6H₂O 1 mg/l) and was adjusted at pH 7.0[3]. Carbon source was added by 1% soybean oil or crude oil into the medium described upper and then incubated at 37°C, 180 rpm for 7 days. The

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culture broth were streaked on LB agar plates containing 1% tributyrin and incubated at 37°C. After then we selected a single colony as bacteria producing oil-emulsifying biosurfactant. Also, the strain DYL-130 was cultured by varying carbon source such as sodium acetate, soluble starch, glucose, maltose, sucrose. Every carbon sources are added by 0.1% in LB broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl).

Identification of 16S rDNA from the strain DYL-130

To identify DYL-130 isolate, chromosomal DNA was extracted from the cells growth LB by a commercial chromosomal DNA preparation kit (Takara, genome DNA purification of *E.coli*) with minor modification. A PCR reaction amplifying the 16S ribosomal DNA was performed with primers 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAA-GGAGGTGATCCAGCC -3' (Each oligonucleotides was corresponded at numerax 9-27 and 1,542-1,525 from *E. coli* 16S rDNA segmence).

Assay for surface activity

To determine the surface tension, the isolated bacteria were cultured in LB medium for 78 h. After the culture supernatant were collected by between evry 6-12 h and surface tension measured by ring method using the DeNouy Tensiometer (Itoh Seisakusho, Japan). The experiment was independently performed three times and recorded the average of the value.

Isolation of Biosurfactant

We cultured DYL-130 strain in LB medium for 4 days at 37°C, 180 rpm. A cell free culture supernatant containing biosurfactants was obtained by centrifugation at 10,000 rpm for 20 min, 4°C. A purification of biosurfactant was performed by affinity chromatography (DIAON HP20, mitsubishi chemical, samyang). The column adsorbent is a synthetic adsorbents of high porous type. It has hydrophobic surface and suitable organic body which has over 1,500 molecular weight. The biosurfactant of culture medium was eluted with 100% methanol than each elution samples (150 ml per bottle) were collected in seven glass bottles. After then the solvent was removed with a rotary evaporator (EYELA N-1000, Japan) and enrichment sample was dissolved in alkaline water (pH 10). The purified biosurfactant were identified by TLC (thin layer chromatography, silica gel 60 F254, MERCK) with CMW (chloroform/methanol/water = 65 : 25

: 4) as a developing solvent mixture. The purified biosurfactant was tested for biosurfactant activity using the drop-collapsing assay[12]. We dropt 25 µl droplets of each crude biosurfactant and purified biosurfactant (fraction 4) to the parafilm and methylene blue was added to stain the samples for photographic purposes. The methylene blue has no influence on the shape of droplets.

Emulsify activity and stability of biosrfactant from DYL-130

Emulsifying activity and stability were estimated by method of Cirigliano and Carman[4,5]. Emulsifying activity was determined by the addition of equal volumes of hydrocarbons and oils including tributyrin, decane, dodecane, tetradecane, hexadecane, soybean oil, crude oil and kerosene. Next a cell-free culture mixed with a vortex for 2 min. After all, absorbance was measured by UV/visible spectrophotometer (ultrospec 2100 pro, Amersham) at 540 nm every 10 min for 60 min. Later than the log of the absorbance was plotted versus time and the slope (decay constant, K_d) of the line was calculated.

Results and Discussion

Isolation and identification of the strain

We isolated several tens of the bacteria that make use of crude oil as a carbon source from soil in Duck-Yu Mountain and an effective biosurfactant-producing strain, DYL-130, was isolated from them. This strain was isolated and selected among based on the lowest surface tension by its culture supernatant and TBN-plate activity. The TBN-plate is LB broth by containing 1% tributyrin This strain is a gram-positive and rod-shaped bacterium by SEM (Field Emission Scanning Electron Microscopy, data not show). To identify the strain, PCR amplifying the 16S ribosomal DNA and be described at materials and methods gene was performed. The sequence analysis of the resulting PCR fragment (respectively 1.5 kb, 980 bp) showed the highest homology (99%) with the 16S rDNA of *Bacillus atrophaeus* (AB021181, AF272016). Thus we named to *Bacillus atrophaeus* DYL-130. *Pseudomonas* sp., *Aeromonas* sp., *Acinetobacter* sp., *Klebsiella* sp. and *Arthrobacter* sp. were almost reported until now as : degrading oil and producing emulsifier strains[3,13,17,20].

Surface activity of crude biosurfactant from DYL-130

It was assumed that the biosurfactant from *Bacillus atro-*

phaeus DYL-130 might be secreted from the cell possible of decreasing surface tension LB medium. The surface tension by the supernatant from DYL-130 culture was measured using the DeNouy Tensiometer (Itoh Seisakusho, Japan). As shown in Fig. 1, it was shown that in supernatant of DYL-130 the surface tension is reduced by 28 mN/m when compared with sterilized LB medium and reduced surface tension was continually maintained until 78 h. The critical micell concentration (CMC) value is defined as that point at which isolated surface-activity compound no longer aggregates to form micelles[12] and the CMC of crude biosurfactant was 35 mg/l (data not show). This results is similar to reported biosurfactant, since those of *Bacillus* sp. as well as *Norcardia* sp. etc. has capability for reducing surface tension range of 25 to 40 mN/m[3,14,15]. Fig. 2 shows that

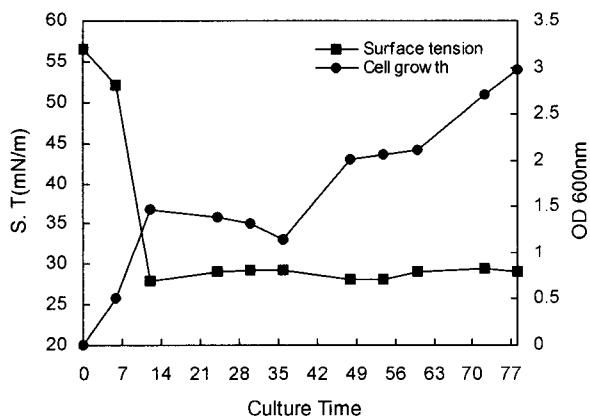


Fig. 1. Change of cell growth and patterns of biosurfactant production by cultivation times. The *Bacillus atrophaeus*. DYL-130 was grown in LB medium at 37°C, 200 rpm for 78 h.

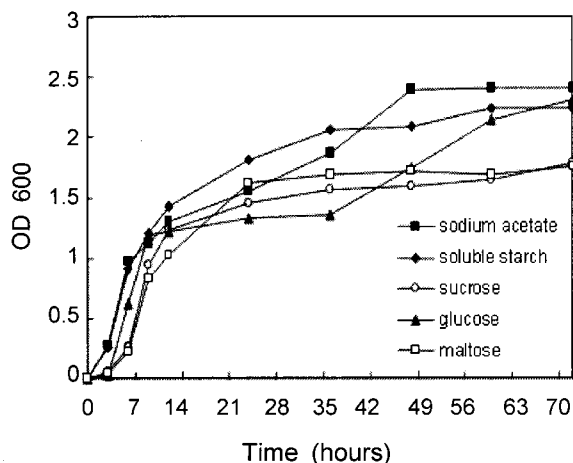


Fig. 2. Production of biosurfactant using various carbon sources.

The biosurfactant was produced using various carbon sources and the surface tension was decreased to 28 mN/m in these carbon sources.

DYL-130 use a variety of carbon sources for growth, including glucose, maltose, sucrose, soluble starch, sodium acetate. The DYL-130 was grown by acetate and soluble starch more than others. The surface tension decreased to 28-29 mN/m in all carbon sources. So, the biosurfactant would be produced using various carbon source.

Emulsifying activity and stability

We used as a crude biosurfactant when surface tension could be completely decreased by the supernatant at 24 h of DYL-130 culture. In the Fig. 3, the biosurfactant was strongly emulsified kerosene (2.88) but in case of stability, soybean oil was better than kerosene (Table 1). Also it shows that emulsifying activity is on a tendency to powerful according

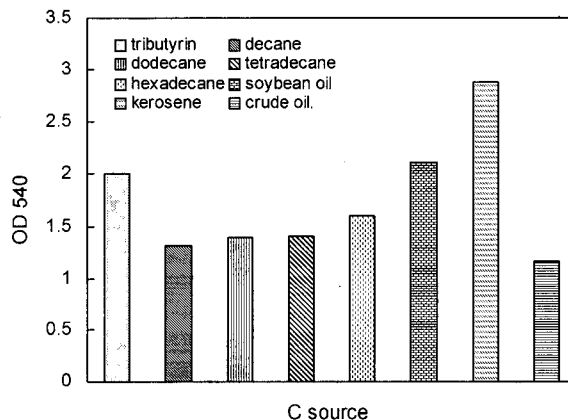


Fig. 3. Emulsification activity of various substrates by the crude biosurfactant solution.

The sample mixture was shaken vigorously by vortexing. The absorbance (A_{540}) of the emulsion was determined after the 10 min.

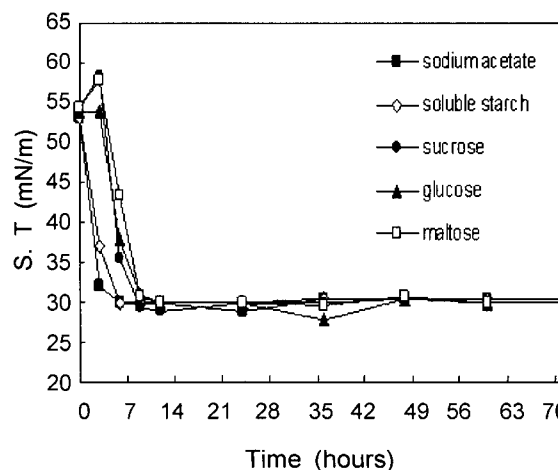


Table 1. Emulsification activity and stabilization of various substrates by biosurfactant solution

| Substrates | Emulsification activity (OD ₅₄₀) ^a | Decay constant (K _d , 10 ⁻³) ^b |
|-------------------------------|---|--|
| Soybean oil | 2.10 | -0.00 |
| Kerosene | 2.88 | -11.32 |
| Tributylin | 1.99 | -9.56 |
| Crude oil | 1.16 | -2.21 |
| Hexadecane(C ₁₆) | 1.59 | -3.18 |
| Tetradecane(C ₁₄) | 1.40 | -5.08 |
| Dodecane(C ₁₂) | 1.39 | -3.65 |
| Decane(C ₁₀) | 1.30 | -5.34 |

^aThe emulsification assay was performed in the presence of biosurfactant as described in the text. After the initial 10 min holding period, absorbance readings were taken every 10 min for 60 min.

^bThe log of the absorbance was then plotted versus time and the slope (decay constant, K_d) of the line was calculated.

as lengths of carbon chain increase. These results are opposed to biosurfactant produced by *Pseudomonas aeruginosa* [6]. Stability of activity indicated as K_d which is a decay constant. We compared our biosurfactant with different chemically synthesized surfactants (Table 1). As a result, we confirmed that Tweens and Triton X-100 have similar activity and stability. The biosurfactant from DYL-130 was more effective than Spans and SDS (Fig. 4) and it is very alike biosurfactant from *Norcardia* sp. L-417[14]. Therefore these characteristics of our biosurfactant indicates that it could be potentially substituted for chemically synthesized surfactants.

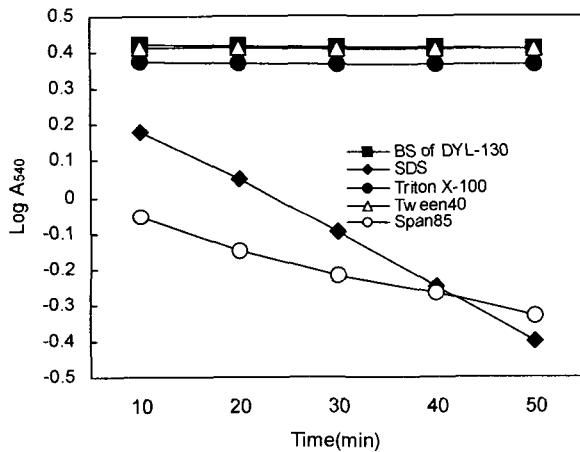


Fig. 4. Comparison of emulsion stabilization between the biosurfactant and various chemical surfactants.

Emulsifying substrate was soybean oil. The absorbance (A₅₄₀) of the emulsion was determined at the indicated times. After the initial 10 min. holding period, absorbance readings were taken every 10 min. The log of the absorbance was then plotted versus time.

Characterization of isolated biosurfactant

We isolated the biosurfactant producing DYL-130 by column chromatography and the isolated material revealed following characterization. Fig. 5 shows the thin-layer chromatography of crude biosurfactant (Fig. 5-A) and purified biosurfactant (Fig. 5-B) with affinity column chromatography. We used sterilized water to the surface-activity substance as a development agent. It was confirmed that the R_f value of the biosurfactant was 0.78 (Fig. 5-A). After the purification, we also confirmed same value (Fig. 5-B). This result is a similar to lipopeptide producing *Bacillus subtilis*[15]. Accordingly, in the future, we will have to perform the analysis of structure of biosurfactant from DYL-130. In fractionated samples, fraction 4 was tested in drop-collapsing assay for surface activity (Fig. 5-C). The results show that

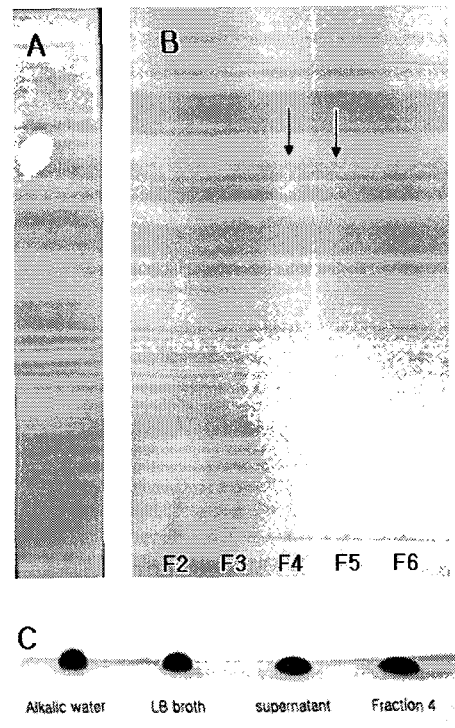


Fig. 5. Isolation of biosurfactant by column chromatography (DIAON HP20) and the assay of biosurfactant activity using drop-collapsing test.

A, Identification of crude biosurfactant. : We filtered the culture supernatant of DYL-130 with 0.45 μm filter (Minisart, sartorius) and identified by TLC (thin layer chromatography, silica gel 60 F254, MERCK) with CMW (chloroform/methanol/water = 65 : 25 : 4) as a developing solvent mixture. B, Identification of purified biosurfactant by column chromatography. : The isolated sample (Fraction2 - Fraction6) was identified by TLC. C : Biosurfactant activity of culture supernatant and fraction 4 of *Bacillus atrophaeus* DYL-130.

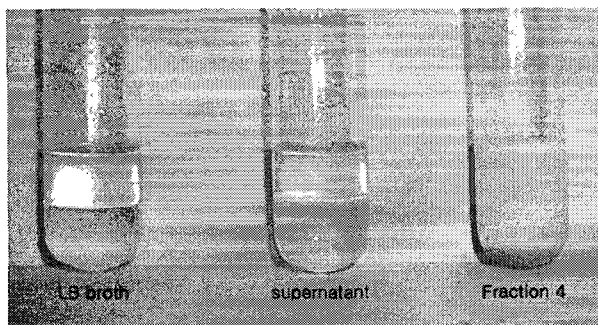


Fig. 6. Comparison of the emulsifying activity with culture supernatant and purified sample.

An equal volume of toluene was added to supernatant, fraction 4, LB broth and mixed in glass vials. The layers represent the stabilized emulsions after 1 h of incubation.

the alkaline water and LB broth did not spread on a hydrophobic support (parafilm), whereas the crude supernatant derived from the strain DYL-130 and fraction 4 did.

To determine the emulsifying activity of fractionated sample, an equal volume of toluene was added to the crude supernatant, fraction 4 and LB broth (control) and mixed in glass vial for 2 min (Fig. 6). Next the mixture was incubation at room temperature for 1 h. We observed that the control (LB broth mixture) and supernatant divided two layer. The upper floor is a toluene layer, and the low floor is a water layer. In case of supernatant, the water layer is more turbid than control (LB broth). In addition, it shows the presence of water/toluene emulsion at the interface of two layer and this results are similar to *Pseudomonas putida* lipopeptide biosurfactant, Putisolvin I, II [12]. In the fraction 4 mixture, we confirmed that the mixture indicated almost water/toluene emulsion and the emulsion maintained over the three hours. Consequently, the isolated biosurfactant from DYL-130 has very powerful emulsifying activity and it is possible to substitute an friendly environmental emulsifier for chemically synthesized emulsifier.

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초록 : *Bacillus atrophaeus* DYL-1300이 생산하는 biosurfactant의 분리 및 특성

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원유 분해능이 강력한 균주를 얻고자 덕유산의 토양으로부터 crude oil을 탄소원으로 이용하는 수십 종을 분리하였다. 분리된 균주 중 원유분해능 및 biosurfactant 생성능이 우수한 균주를 선별하여, 형태학적 특성을 관찰하고 16S rDNA sequence와 *gyrA* gene sequence를 통하여 *Bacillus atrophaeus* DYL-130으로 동정하였다. 동정된 균주 배양액의 표면장력은 최저 28 mN/m까지 감소되었다. 또한 *Bacillus atrophaeus* DYL-130이 생산하는 biosurfactant가 column chromatography에 의하여 분리 되었으며, 분리된 biosurfactant의 toluene 유화능 및 crude biosurfactant의 유화활성과 안정성이 시험 되었다. Crude biosurfactant의 유화활성은 kerosene에서 최대였으며, soybean oil에서도 높은 편이었으나 유화안정성의 경우 soybean oil을 기질로 하였을 경우가 kerosene을 기질로 하였을 때 보다 우수하였다. 또한 crude biosurfactant의 유화안정성을 합성계면활성제와 비교 한 결과 DYL-130이 생산하는 biosurfactant의 유화안정성이 합성계면활성제와 유사하거나 뛰어남을 확인하였다. Column chromatography에 의하여 분리된 biosurfactant는 drop-collapsing method에 의하여 surface-activity가 확인되었으며 또한 toluene에 대한 유화력이 매우 뛰어난 것을 확인 하였다. 따라서 DYL-130에서 추출한 biosurfactant는 합성계면활성제를 대체할 수 있는 환경친화적인 생물 계면활성제로 사용될 수 있는 가능성을 보여주고 있다. 산업적으로 이 물질을 이용하기 위해서 *Bacillus atrophaeus* DYL-130이 생산하는 biosurfactant에 대한 구조분석과 물리 화학적 특성, 생분해도 및 환경독성 등의 조사를 수행하여야 할 것으로 생각된다.