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DIESEL OIL UTILIZING -BIOSURACTANT PRODUCING BACTERIAL ISOLATE AND CHARACTERISTICS OF PRODUCED BIOSURFACTANTS TOWARD BIOREMEDIATION APPLICATION

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Abstract

A bacterial strain was isolated from Saigon River and showed its ability of utilization diesel oil (DO) as the main carbon source. Hydrocarbon degradation could reach 80% within 5 days of liquid cultivation under the optimal condition. At the late log-phase of growth in DO oil, these bacteria produced a mixture of biosurfactants, which lowered surface tension of cultured broth to 31.2mN/cm^2, and made a substantial emulsification activity with xylen and n-hexane. The effects of salt concentrations and pH to biosufactant producing were also studied. The strain was identified as *Pseudomonas aeruginosa.*

Cell-free-cultured broth showed a strong releasing DO from oil-bound sandy by thousands time lower concentration in comparison with SDS, a chemical surfactant, and significantly promoting DO utilization by the same strain. The results indicate that it is possible to apply this type of crude biosurfactant to the bioremediation of hydrocarbons.

Key words: Biosurfactant, Diesel oil degradation, *Pseudomonas aeruginosa.*

1. Introduction

Biosurfactants are surface active substances produced by living cells. They have the properties of reducing surface tension, stabilizing emulsions, promoting foaming and are generally non-toxic and biodegradable.

Microorganisms which synthesized biosurfactants can be bacteria, yeasts or fungi. Among of those, the bacteria have been intensively studied so far [1 & 2] and their biosurfactants products are mostly glycolipids (rhamnolipids or sorphorolipids), lipoproteins (surfactins) or natural lipids. These substances are widely used in the industry and environment.

Toward bioremediation of hydrocarbon pollution, biosurfactants are considered to enhance hydrocarbon biodegradation processes by several ways [2, 4, 5, 6, 7] : (i) to increase morbidity of hydrocarbons especially low dissolve hydrophobic substances in water or soil through forming micelles; (ii) to facilitate the cell adhering to the hydrophobic surface, (iii) to increase cell surface hydrophobicity at low concentration. These properties are important for application practice.

In this study, we have isolated a bacterial strain, namely SG, from Saigon River. The strain showed ability of utilization of diesel oil and during biodegradation it produced a mixture of biosurfactant. Diesel oil degradation and biosurfactant production was investigated by effects of factors such as medium pH, salt concentration. Cell free cultured broth (containing SG7 biosurfactant) was used to study effects to oil release from oil bound sandy and biodegradation of oil by the same strain.

2. Experimental procedure

Materials and Chemicals: Gram staining solutions, SDS (Sodium dodecyl sulfate), PCA agar medium were from Merck, Germany. API -20 NE test kit and analysis software were from BioMerieux,USA . Blood-agar-plates were provided from Pasteur Institute in Hochiminh city. *Pseudomonas aeruginosa* ATCC 27853 reference strain was from NamKhoa Co., Vietnam.

Bacterial isolation and growth: Water samples was collected from Thuy-Doi shipping yard belonging to Sai-Gon River and spread on the blood-agar-plates (containing 5% sheep blood) and incubated at 35 ^{o}C. The colony with a hemolytic zone surrounding was selected on PCA agar (Merck) plate. Single strains then were subjected for DO-oil utilization and biosurfactant activity tests. Liquid mineral medium containing 0.7 g/l Na_2HPO_4, 0.3 g/l KH_2PO_4, 3g/l KNO_3, 0.4g/l $MgSO_4$ and 30 % (v/v) of filtrated river water, pH 7.2, was used. When indicated the medium will be added with DO oil or/ and NaCl to desired concentrations.

Morphological study was studied by microscopic observation of fresh and alive cells. Gram staining was followed method of Merck producer and judged under microscopy. Catalase activity was tested using H_2O_2 solution dropped on the surface of the bacterial biomass.

API -20 NE Test for SG7 was followed strictly by a protocol provided by BioMerieux,USA.

16S rRNA analysis : SG7 strain was sent to Biotechnology Center, Vietnam National University-Hanoi for 16S rRNA sequence verification (ABI PRISM® 3100-Avant Genetic Analyzer) using PCR primers: 27F <5'-AGAGTTTGATCCTGGCTCAG>; 1525R <5'-AAAGGAGGTGATCCAGCC>; 780F <5'- GAATTGATACCCTGGTAG>; and 920R 5'-GTCAATTCCTTTGAGTTT-3'.

DNA-DNA Hybridization: Intensively, about 300 mg of biomass of each SG7 and the reference ATCC27853 strain was prepared from growth in LB medium for 20 hours at 30°C. DNA extraction was done by cell lyses and purified by $CsCl_2$ gradient ultra centrifugation at 100.000 rpm at 15°C for 16hrs. Full length biotinlated and activated DNA-DNA hybridization was performed at 55°C for 2-3 hours and read through Microplate Reader at 450 nm.

Oil dispersion test: The test was performed in a clear glass petri Φ 10cm plate. Diesel oil was spayed to form a fine and thin layer of oil on distilled water and hold for stationary about 20 minutes. 10 µl of biosufactant containing solution was vertically added onto the center of the oil layer. The diameter of the dispersed oil layer spot was immediately measured.

Emulsification test (E_{24}): 2ml of cell-free-supernatant was mixed with 2 ml of xylen (or n-hexane or diesel oil) and 3 ml of phosphate buffer pH 7.6 containing 1mM $MgSO_4$ in a glass 1cm x 15 cm tube by vortexing for 1 minute at 2000rpm. After 24 hrs holding at room temperature, the percentage of volume occupied by emulsion was measured.

Hydrocarbon measurement : The amount of diesel oil remaining in cultured broth was measured by weight in a corning 15ml centrifuge tube. Evenly 5-10 ml of the mixed well cultured broth was de-emulsified by adding ethanol kept frozen for several hours. After thawing, the tube was centrifuged at 3500rpm and the oil layer was carefully removed and weighted.

Diesel oil release from oily sandy: 100 gram of sand was washed well and dried at 105°C overnight to remove water completely. After cooling to room temperature, sand particles were mixed well with 20 ml of diesel oil. For testing, 15 gram of oily sandy was put in the bottom of a glass 1cm x 15 cm tube, 5 ml of biosurfactant containing solution was gently added again the wall of the tube. Cell free supernatant or 1% SDS with dilution factors from 1 to 10^7 was used. The height of oil layer raised from bottom was measured after holding at RT for 24hrs.

Experimental design for the effect of added biosurfactant in DO oil degradation: Test was performed in 300 ml glass flask containing 100ml mineral medium, 18 ml of sterilized diesel oil and 1 ml of the cell-free-supernatant with dilution factors from 1 to 10^7. SG7 cells were harvested from one overnight growth on a PCA plate and used to make cell suspension by a mineral medium and made in 1ml aliquots for each growth test flask. Culture was shaken at 200 rpm at RT and at each indicated time 10 ml of the mixture was withdrawn and the diesel oil containing in a sample was measured by weighing method as described above.

3. Results and discussion

3.1. Identification of bacterial strain SG7

From 7 strains isolated by their hemolytic activity on blood- agar plate and showed diesel oil utilization, only one strain, namely SG7, strain produced a highest biosurfactant/ emulsification activity when growth by diesel oil. The SG7 cell appeared as a short rod shape and mobilized. Cells when grown on PCA agar formed a round and medium sized -whitish colony and excreted green pigment. On the mineral agar medium with sprayed oil, cells grew slower and forming smaller and more transparent colonies. The strain is Gram negative (-) and it could show catalase but not fermentative activity. These characteristics can say that the strain likely belong to *Pseudomonas* genus.

Table: Results of API-20NE tripping test *(reading after 48 hr of incubation)*

48h	±	-	-	+	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	+
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	NGT	CAP	ADI	MLT	CIT	PAC	OX
ID No.	**1**			**3**			**5**			**4**			**7**			**7**			**7**		

The biological API-20NE test results showed that SG7 strain had a high similarity, 99.8 %, to *Pseudomonas aeruginosa* (Table). This kind of test is one of the most convenient tests to identify pseudomonad; however, the interpretation based on test result sometimes caused an error due to effect of cell aging and testing performance.

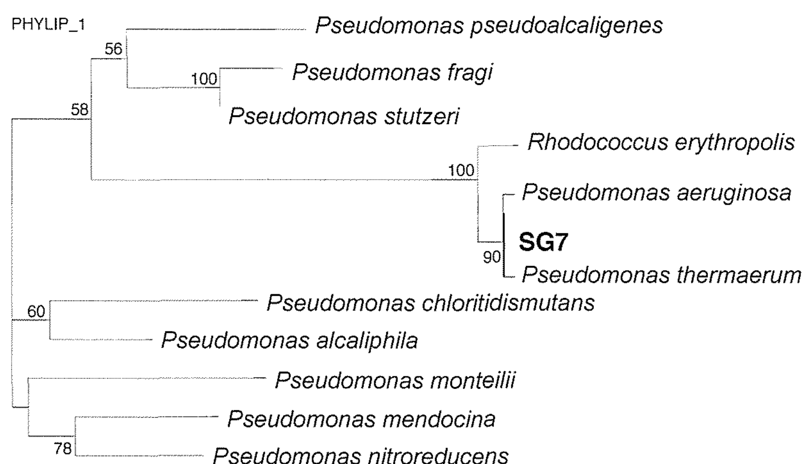

Fig. 1. Phylogenetic tree. *Similarity of SG7 gene to others in GenBank was estimated by BLAST system.*

16S rRNA sequence homology of SG7 strain was further studied using specific PCR primers to amplify a high consensus area of the bacterial target gene. DNA homological searching using BLAST was identified SG7 as *Pseudomonas aeruginosa* 99,7% (1446/1450) or *Pseudomonas thermaerum* 99,8% (1447/1450) (Fig. 1). The confident identification was obtained from the result of DNA-DNA hybridization experiment, in which full length chromosomal DNA of SG7 and *Pseudomonas aeruginosa* ATCC 27853 were hybridized to each other by nearly 100%. SG7 is not new strain, since *Pseudomonas aeruginosa* species were known as one of the most common bacteria in the environment. Many of them showed ability to degrade saturated or aromatic or even

chlorinated hydrocarbons [3, 4, and 5]. *Pseudomonas aeruginosa* ATCC 27853 was also reported by its capacity of hydrocarbon degradation [7].

3.2. SG strain utilized diesel oil as the main carbon sources

In the liquid culture, SG7 could degrade diesel oil having carbon chain ranging from C_9 to C_{31} (data not shown data) without specific selection of carbon chain number. The efficiency was ranged from 70-92% (Fig. 2). Number of cells reached maximal value after about 2 days of cultivation. The hydrocarbon degradation started with a short lag of about 6 hrs if cells were picked up from PCA agar plate or presented an immediately log phase if cell were pre incubated with medium containing oil. Biodegradation ended after about 5days (data not shown). Diesel oil utilization was slightly effected by pH change. Biodegradation highest at initial slightly alkalic pH 8.0- 9.0 and reduced about half yield when pH 5.0-6.0 employed. Growth was not inhibited when the oil concentration up to 30 % was used at starting (data not shown).

Fig. 3 shows the effect of NaCl on hydrocarbon utilization. The strain gave the highest oil biodegradation when grown in 0.8% NaCl or in very low salt concentration. This is unsurprised because SG7 strain was isolated from Saigon Harbor where the river water is slightly brackish water. It is interesting that under 4% of salt, this level was higher than that in any seawater along Vietnam coastal line; cells still gave about 40% biodegradation yield.

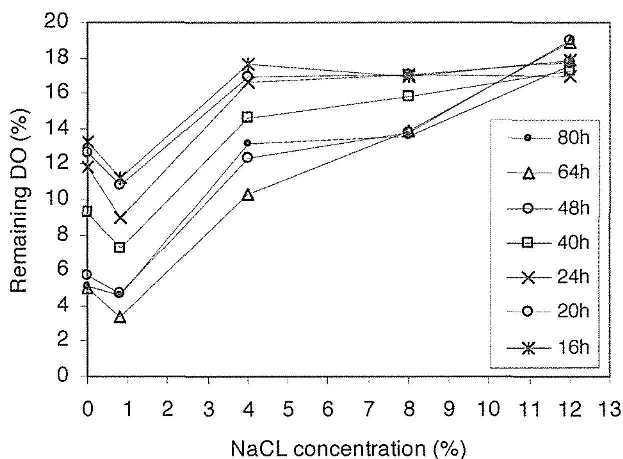

Fig. 2. Effect of medium pH on diesel oil biodegradation. *Started oil concentration was 18% in 100 ml mineral medium pH 8.0, shaken at 200 rpm at RT. The values presented on 0 % NaCl lane actual was less than 1 g/L. Values were determined by weighing method.*

3.3. Characteristics of the produced biosurfactant

At the late log phase of the biodegradation, SG7 strain started produce surfactant activity which could be followed by oil dispersion tests using the cell free culture broth (Fig. 4). Lowest surface tension of the supernatant measured was 31.2 N/cm2. Along with surface activity, the cell free cultured broth also performed an excellent emulsification activity with xylen or n-hexane. E24 for xylen was nearly 100 % (Fig. 3) and that for n-hexane was about 80% (data not shown). Effect of pH on biosurfactant production was similar to that of observed for hydrocarbon degradation, highest at pH 8.0-9.0 and reduced about half of that at pH 5.0-6.0 (Fig.3 and Fig.4). The biodegradation, however, still continued to be produced at the time of 5 days, while number of the cell starting decayed.

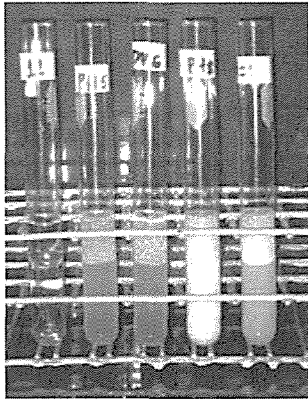

Fig. 3. Effect of growth pH on biosurfactant production (based on emulsification activity). *Xylen emulsification tests used cell free cultured broth under different initial pH values and after 5 days of cultivation. From left to right: Test control; pH 5.0; pH 6.0; pH 8.0; and pH 9.0*

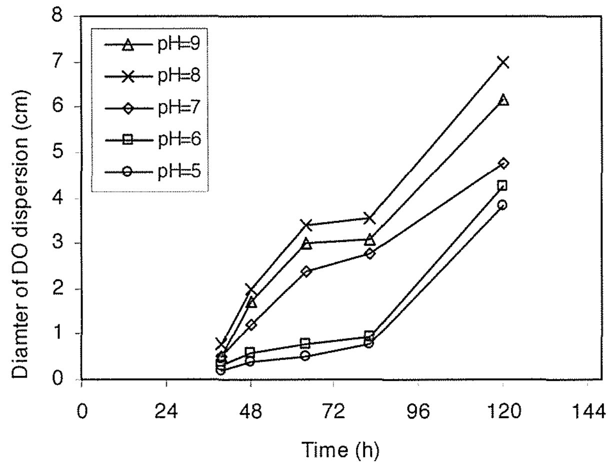

Fig. 4. Effect of growth pH on biosurfactant production (based on surface activity). *Oil dispersion test was conducted by using cell free cultured broth under different growth pH values (from 5.0-9.0) and after 5 days of cultivation.*

High concentration of salt (4-12%) on biosurfactant production, however, produced a longer lag phase of surfactant activity (Fig.5). After 5 days of cultivation, there was not much significant difference on activity along a large different concentration of salt from nearly 0% to 12% (Fig.5). The surface activities measured in case of 12% salt medium seems not due to the accelerated growth, since at this condition hydrocarbon degradation happened with a very slow rate (Fig. 2).

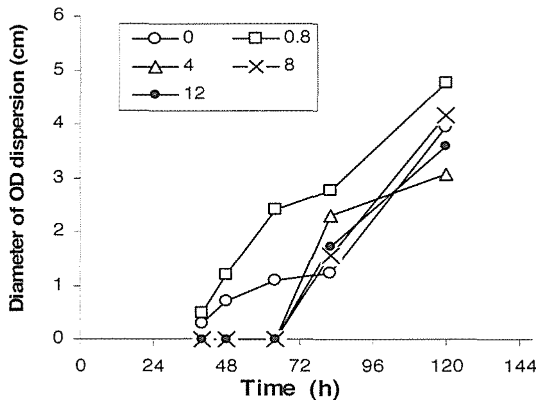

Fig. 5. Effect of growth pH on biosurfactant production (surface activity). *Oil dispersion test was conducted by using cell free cultured broth under different growth pH values (from 5.0-9.0) and after 5 days of cultivation.*

3.4. Effect of added biosurfactant to oil releasing from oil bound sandy

One of biosurfactant applications to bioremediation of contaminated hydrocarbons is to reduce the hydrophobic binding strength of hydrocarbons to soil/ sand particles, so the microdegraders could easier access contaminants. Fig.7 shows the effect of addition of cell free cultured broth with various dilutions into diesel oil previously bound to sand particles. In water environment, SG7 biosurfactant caused oil release from the bottom of test tubes. The amount of released oil represented by the height of oil column raised on top of water was linearly dependent on logarithmic concentrations of surfactant. Fig.7 shows that 0.75% (w/v) crude biosurfactant solution (cell free cultured broth) was thousands time stronger than 1% (w/v) of SDS solution, a chemical surfactant.

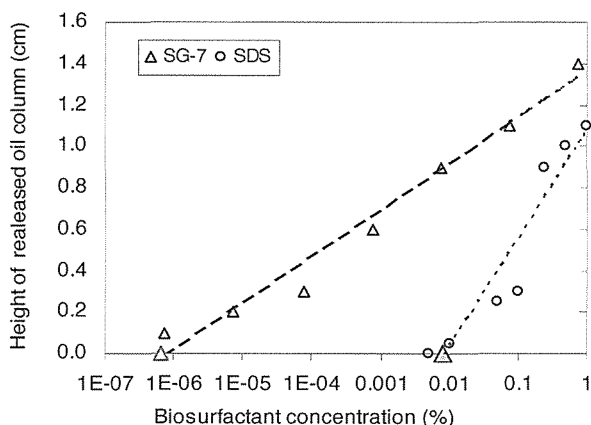

Fig. 6. Effect of added biosurfactant to oil release. *Cell free cultured broth of 5 days cultivation (triangles) and 1% of SDS (circles) with various dilutions were used. The concentrations of this crude biosurfactant were calculated using its dried weight value of 7.5 g per a litter. The height of raised oil column was measured after 24hrs.*

3.5. Effect of added biosurfactants on the biodegradation of diesel oil by SG7 strain

It is assumed that if biosurfactant is produced by the cell to help biodegradation of hydrocarbon, it will effect to biodegradation rate. In the experiment the cells were harvested from PCA plate in order to minimize amount of biosurfactant at the beginning of growth. It seemed that SG7 used very little amount of added biosurfactant for growth (Fig.7). Within 37 hrs incubation, the only growth with 0.1% (v/v) added crude biosurfactant solution showed slightly inhibition as 30% oil remained (5% w/v) while in the other cases oil was almost degraded. This finding indicates that biosurfactant with excess amount needed it may enhance biodegradation at beginning but later on caused an inhibition to complete hydrocarbon degradation.

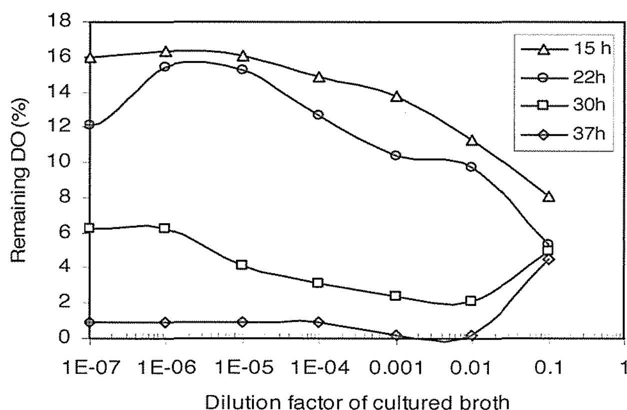

Fig. 7. Effect of added biosurfactant on biodegradation of diesel oil by SG7 strain. *Experiment was conducted by using cell free cultured broth. Each growth flask was inoculated with 10^6 cell/ml of SG7 precultured on PCA plate and 16 % w/v of diesel oil.*

4. Conculsion

SG7 strain belongs to *Pseudomonas aeruginosa*. It is not new specie but presented interesting activities of diesel oil biodegradation and crude biosurfactant production. The question is that what kind of biosurfactant was presented in the cultured broth of strain SG7. Strain showed blood hemolysis on plate it could suggest the biosurfactant belongs to anionic type biosurfactant and generally glycolipids group. In another study we have tried to isolate this kind of surface active compound by thin layer and column chromathography and estimated that biosurfactant was round 1.2 g/l. And further NMR analysis showed that compound having dirhamnolipids structure (Rha-C10 _Rha-C10) and a small amount of monorhanolipid.

Rhamnolipid production by cells depended on medium pH with a similar manner as oil biodegradation, this might indicate that biosurfactant involving in cell itself getting an access to hydrophobic phase.

Biosurfactant is needed at very low concentration to promote hydrocarbon degradation in water. The concentration biosurfactant presence higher than needed may inhibit biodegradation process. This should be considered to optimize amount of biosurfactant use in bioremediation.

Under high concentration of NaCL, bacteria grew very slowly but still produce substantial biosurfactant activity as that of under low salt conditions. It is interesting to study whether there is other type of biosurfactant beside of rhamnolipid produced by SG7 strain during biodegradation of diesel oil.

Acknowlegement

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