

MOLECULAR IDENTIFICATION AND ISOLATION OF CAPNOPHILIC AND MESOPHILIC BIO-EMULSIFIER AND BIO-DEMULSIFIER BACTERIA FROM KHASH MUD VOLCANO

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ABSTRACT

About 40 oil fields in Iran (27 oil fields on land and 13 oil fields in the sea) that are mainly located in the south and south west of the country face with severe emulsion problems. This fact shows the need of demulsifiers and emulsifiers usage. Bio- demulsifiers and bio-emulsifiers (usage of microbial biosurfactant) can be widely used in the oil and metallurgical industry, food industry, and textile industry instead of physicochemical methods. In this study, capnophilic and mesophilic bacterial isolates from Khash mud volcano had been purified and incubated on the *Plate Count Agar medium* (Merck/germany) at 30 ° C, in atmospherer with 10%CO₂ for 24-48 h. Bio-emulsifier isolates with β-hemolyse activity were screened. Isolates were inoculated in erlenmeyer flask including a medium with mineral salt solution (0/03 % yeast extract and glucose) with 1% kerosene and incubated at 120 rpm and 30 ° C for 48-72 h. For bio-emulsifier test, each sample vortexed and assessed their stabilizing emulsification capacity(degree 0-4). In bio-demulsifier test, 1 ml from erlenmeyer flasks added into tubes containing stable emulsions of water / diesel and diesel/water and properly vertexed and incubated in 30 ° c for assessed of demulsification degree (0 to 5). The surface tension of superior isolates were measured by Tensiometer (TD1C LAUDA). Among 25 screened isolates,12 and 10 isolates had bio-demulsification(superior isolate was C₁₁) and bio-emulsification activity(Superior isolate was C₁₈), respectively. Amount of surface tension of superior isolates showed to reduce 27.7 mN/m(Bio-demulsifer) and 22.6 mN/m(bio-emulsifier), compared with the control sample, 40.1mN/m, and were identified as *Bacillus thuringiensis* and *Bacillus anthracis* strain EFF-G51, respectively.

Keywords: Bio- Demulsifier, Bio-emulsifier, Khash Mud volcano, Surface Tension, *Bacillus thuringiensis*, *Bacillus anthracis* strain EFF-G51.

INTRODUCTION

Demulsifiers, or emulsion breakers, are a class of specialty chemicals used to separate emulsions, for example, water in oil (W/O). They are commonly used in the processing of crude oil.¹ About 40 oil fields in Iran (27 oil fields on land and 13 oil field in the sea) that are mainly located in the South and South west of the country face with severe emulsion problems. Most of Iran's crude oil is contaminated 2% to 11% with water and basic sediment which is higher than the international standard (less than 0.5%). So the country pays a

high cost for to abolish the emulsion.² Emulsion breaking and desalination of crude oil in Iran is a combination of injecting demulsifiers (chemicals), heating method and applying the electric current. Demulsifier's type selection is based on Several parameters, much of this material is provided from imports and basically, the criteria for their selection are their performance in the crude oil desalination and the prices offered by suppliers. It seems that the Environmental side effects of byproducts are not considered.³ Due to the large amounts of water, salts and other solids in crude oil, It is necessary to separate these materials from crude oil. Because these impurities cause the operational procedures

and export of crude oil to not have the necessary indicators of refining and transportation and also cause not to be economically efficient.⁴ The presence of salt in the crude oil with water, causes these materials to convert into acids, Specially the hydrochloric Acid which leads to corrosion in refinery installations.⁵ The other method that used to solve these problems is biosurfactants(surface active compounds). These compounds produced with a wide range of microorganisms. Some of the Significant potential applications of biosurfactants are emulsification, demulsification, inhibition of corrosion and reduce viscosity(for heavy oil), therefore biosurfactants can be used instead of many types of chemical surfactants.⁶ The main benefits of using microbes in these industries are their structural diversity, high performance, ecological and physical properties safety, production from cheap raw materials and potential for changes in productive microorganisms (By genetic engineering ,through Biological and biochemical techniques) and their degradability⁷, Some examples of bio-demulsifier bacteria are *Nocardia amarae*, *Corynebacterium petrophilum*, *Rhodococcus erythropolis*, *Torulopsis bombicol* and one Species of *Alcaligenes .sp* and bio-emulsifier are *Pseudomonas sp.* , *Bacillus sp.* and etc.^{4,7-8}. As one of the natural resources for isolation of microorganisms with these abilities, oil sands tailings ponds can be noted that microorganisms have been widely adopted in these areas like Mud volcano areas in Iran that have been formed on oil sands tailings ponds . As Important Mud volcano areas in Iran, one Mud volcano area in Khash port in southern Iran can be noticed.² In screening of bacteria with Bio- demulsification ability, in research carried out in 2014, two isolateds ; *P. aeruginosa* 78 and 90 separated from petrochemical contaminated soils with bio- Demulsifiry ability, which reduced surface tension from 73dyne/cm² to 29dyne/cm² and 33dyne/cm².⁹ Tambekar.D.H.et.al in 2013, they were focused on bio-emulsifier producing *Pseudomonas aeruginosa* isolated from hydrocarbon contaminates soil. From 30 hydrocarbon contaminated soil samples,6 strains of *Pseudomonas spp.* Were isolated with ability of bio-emulsifier and reduction in surface tension (35.97, 63.08, 43.12, 44.09, 31.6, 33.54 mN/m,respectively).¹⁰ The aim of this study was isolated and identify molecular capnophilic and

mesophilic bacteria with Bio- Demulsifir and Bio-emulsifier abilities from Khash Mud volcano area to use of top strains in troubleshooting problems with water/oil emulsions.

MATERIALS AND METHODS

Isolation and purification of capnophilic and mesophilic bacteria from Khash Mud volcano

Type of the study is a descriptive-sectional observation using easy non-random method. 25 samples from main span of mud volcano collected by Sterile cylinder and transported to the laboratory in sterile conditions in the cool box with 4 ° C for research studies. For screening bacterial isolates from samples, serial dilution in sterile condition conducted by 10-10 method to reach the dilution of 10⁻⁵ and from each dilution inoculation was carried out through linear method in sterile condition onto the surface of the Plate Count Agar (PCA) medium (Merck/Germany) to obtain a single colony and incubated at 30 ° C in atmospherer with 10%CO₂ for 24-48 hours(h). Pure bacterial colonies separated in terms of colony's morphological characteristics and to ensure purity of isolates, they incubated at same conditions that noted in above.³

Assessment of Bio-Demulsification activity

First, each of strains inoculated in erlenmeyer flasks including 50 ml of Mineral salt solution(MSS) (0/03 % Yeast extract and Glucose) with 1% Kerosene were mixed and incubated at 120rpm in 30 ° C for 72 h. Then For every bacteria water/diesel and diesel/water emulsions Prepared in the following way:

- 1) Tween 80(0.2 gr)+ 250 ml Distilled water
- 2) Span 60 (0.2 gr)+ Diesel 250 ml

To prepare a stable emulsion diesel / water:

3/5ml(1)

1/5ml(2)

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3/5ml(2)

After preparing emulsions stirred them strongly To create a stable emulsion. Then from samples inoculated bacteria in Mineral salt solution, 1 ml was added to the tubes containing the emulsion and Mix well with Vertex and the results were evaluated according to Table 1.

Table 1
amount of demulsification based on Change in stability of emulsions

Properties	amount of demulsification
No change	0
change in size and amount	1
able to separate 25% water from oil	2
able to separate 50 % water from oil	3
able to separate 75 % water from oil	4
able to separate 100 % water from oil	5

Then the surface tension determined by Tensiometer (TD1C LAUDA).^{3,11}

Assessment of Bio-emulsification activity

This test was based on suggestion method of Francy et al.¹² For primary identified this strains, at first, isolates were inoculated on Blood agar (Merck/germany) plates containing 5%(v/v) sheep blood and incubated at 30°C for 24h(selected beta(β)-hemolyse strains). Selected isolates in first

step inoculated in MSS medium and incubated at 120rpm, 30°C for 48-72h, then samples were vortexed well and assessed their stabilizing emulsification capacity(based on Figure 1). The surface tension of samples were determined by Tensiometer (TD1C LAUDA).^{6,12}

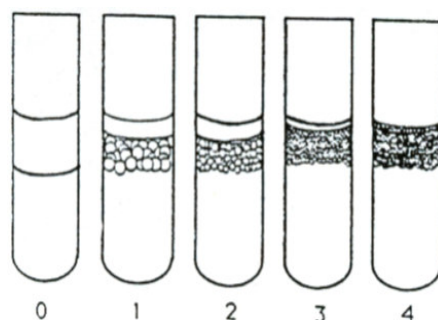


Figure 1
Degree of emulsification

Microscopic and biochemical identification of superior strains

Microscopic identification of the superior strains was done by Gram stain. Biochemical identification was done based on the Colored Atlas book for *Bacillus* spp written by Jnyfr.am.par.¹³

Molecular identification of superior strain

The superior strains were identified by 16S rRNA gene sequencing. Extraction genom was done by the kit. The 16S rDNA was amplified by using universal primer of 27F and 1492R which amplify a 1500-base pair region of the 16S rDNA gene. Primers used for the amplification are forward (27F)(5' AGA GTT TGA TCC TGG CTC AG 3') and reverse (1492R) (5' CGG TTA CCT TGT TAC GAC TT 3'). The amplified DNA was visualized by gel electrophoresis and sequenced. The Chromas LITE was used to analysed 16S rDNA sequence, the most similar bacterial species was found in the GenBank by using BLAST search. Neighbor joining phylogenetic trees were constructed based on 16S rDNA sequences using ClustalW.

RESULTS

Separation and purification of aerobic mesophilic bacterial strains from Khash Mud volcano area

Among 25 mud samples, 25 strains of capnophilic and mesophilic bacteria were isolated and purified.

Assessment Bio- Demulsification activity of isolates

Regarding to Bio- Demulsification activity, c11 strain(superior strain) among 12 isolates with Bio-demulsification activity, based on Table 1 shown 5 amount of demulsification within the shortest time possible and also compared to the control sample, it was able to reduce the surface tension to 27/7 mN/m in compared with surface tension of control (40/1 mN/m).

Assessment Bio-emulsification activity of isolates

Among 25 strains, 10 of them have β-hemolyse activity and among these, 5 strains have bio-emulsifier activity and one of them (C₁₈) have degree 4 for it's bio-emulsifier activity(based on

Figure 2-1).(Figure 2, 3). The C_{18} was able to compared with surface tension of control (40/1 reduce the surface tension to 22/6 mN/m in mN/m).

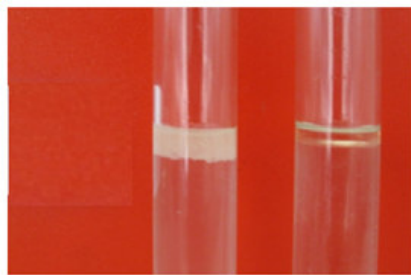


Figure 2
Control negative(at right), Superior strain(C_{18}) (at left).

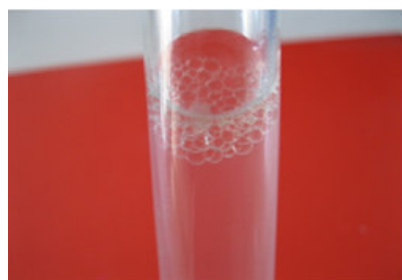


Figure 3
Superior strain(C_{18})

Microscopic and biochemical identification of superior strain

Due to superior strains's microscopic observation a Gram-positive bacilli with spore(C_{11}) and a Gram-

positive bacilli with endospore was found. Biochemical test results expressed based on Table 2 for C_{11} and Table3 for C_{18} .

Table 2
Results of biochemical tests of C_{11} strain

Test \ Strain	Anaerobic growth	Motility	Acid fast staining	LV reaction	Citrate utilization	VP reaction	Growth in 7%NaCl	Starch hydrolysis	Indol	Gelatin hydrolysis
C_{11}	+	-	-	+	+	+	+	+	-	+

(+): Positive, (-): Negative.

Table 3
Results of biochemical tests of C_{18} strain

Test \ Strain	Oxygen	Motility	Oxidase	Catalase	LV reaction	VP reaction	OF (D-Glucose)	Starch hydrolysis	Indol	Gelatin hydrolysis
C_{18}	F	-	-	+	+	+	+	+	-	+

F:Facultatively anaerobic. (+): Positive, (-): Negative.

Molecular identification of superior strain

On the basis of multiple sequence alignments to rooted phylogenetic tree with branch length (UPGMA) of 16S rDNA sequence by CLUSTALW, the strain C11 exhibited high level (99%) and C₁₈(96%) of similarity with the known sequences in the public databases in NCBI and BLAST results, C₁₁ was identified as *Bacillus thuringiensis* and C₁₈ was identified as *Bacillus anthracis* strain EFF-G51.

DISCUSSION

In this study evaluated Bio- Demulsifier and Bio-emulsifier activity of capnophilic and mesophilic isolates collected from Khash mud volcano area in southern Iran. Among 25 isolates, 12 isolates and among these, C11 strain showed highest amount of Bio- Demulsification Activity based on Table1. Demulsification amount of C11 was 5, Which suggests that 100% of the ability to separate water from oil within the shortest time possible. Also during the analysis of the surface tension done by Tensiometer, the superior strain (C11) Compared with the control sample surface tension that was 40/1 mN/m and showed the ability to reduce surface tension closed to half amount (27/7 mN/m) of this factor in control sample that is significant. Due to the microscopic, biochemical and molecular identification superior strain was *Bacillus thuringiensis*. In research conducted in 2014 during Screening bacteria with Bio-Demulsification ability, two isolates of *Pseudomonas aeruginosa* 78 and 90 with Bio-Demulsification ability, separated from petrochemical contaminated soils which reduced surface tension from 73 dyne / cm² respectively, to 29 and 33 dyne / cm².⁹ In another study in 2013, Bio- Demulsification activity of *Paenibacillus alvei* ARN63, breaking heavy crude oil emulsion in water examined and this strain showed the ability to reduce surface tension from 58mN/m to 24/7mN/m.¹⁴ Also in studies that mentioned earlier compared with this study Surface tension was reduced to almost half amount of the surface

tension of control sample. Due to the mentioned strains isolation sources in two studies conducted that noted before which were petrochemical contaminated areas, it is confirmed that *Bacillus thuringiensis* isolation source in this study in Khash Mud volcano Located in the petroleum ponds of southern Iran are appropriate resources. In studies have been don in 2013 and 2014 proved the Bio-Demulsification activity in *Bacillus mojavenensis* XH-1¹⁵ and *Bacillus cereus*¹⁶ which conform widespread Bio- Demulsification activity in the genus *Bacillus* and it is noted that in our study the superior strain determined *Bacillus thuringiensis*. There was no studies found in the field Bio-Demulsification activity of *Bacillus thuringiensis* but there is one study in this field that had been done in 2014 and Bio- emulsification combination with Antifungal activity from *Bacillus thuringiensis* pak2310 was produced and it's production conditions optimized.¹⁷ About Bio-emulsifier activity in this study, among 5 strains with Bio-emulsifier activity, C₁₈ have degree 4 for it's activity and was able to reduce the surface tension to 22/6 mN/m in compared with surface tension of control (40/1 mN/m). The superior strain was identified as *Bacillus anthracis* strain EFF-G51. Gudina et al.¹⁸ evaluated Bio-emulsifier activity of *Paenibacillus* sp. strain from crude oil that produced a lipopeptide biosurfactant able to reduce the surface tension up to 35 mN/m. 19. Viramontes-Ramos et al.¹⁹, among 324 bacterial strains were tested, 17 of them were positive for the drop-collapse and hydrocarbon-layer agar methods. Most of them were *Pseudomonas* and it's surface tension were in the range of 58 to 25 (mN/m). Superior strain of this study in compared to strains were noted in tow articles noted above was the most reduced surface tension. No study didn't mention to Bio-emulsifier activity of *Bacillus anthracis*. According to this article it is recommended that future studies producing condition of Bio-Demulsifier and Bio-demulsifier by the strains were obtained in this study will be optimized and antimicrobial activity of their biosurfactants will evaluate.

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