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ISOLATION, SELECTION AND MOLECULAR IDENTIFICATION OF BIOSURFACTANT-PRODUCING EXTREMOPHILICBACTERIES FROM CRUDE OIL POLLUTED SOIL

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Abstract

Ten bacterial strains with hydrocarbon degrading capacity were isolated from a soil sample that had been polluted with crude oil from the area of Surgut in Russian Federation. This area is characterized by its long winters, with an annual average temperature of -1.7ºC. The isolated bacterial strains live and thrive at a very low temperature, which makes them ideal to be used in unfavorable environmental conditions for the majority of surfactants.In order to evaluate the biosurfactant production of these strains, the following methods were used: surface tension measurements, drop dispersion, hemolytic capacities assay and emulsification rate assay.These analysis showed that two of the strains, *Bacillus subtilis* and *Klebsiellaoxytoca,* was efficient biosurfactant producers. The surface tension decrease when using *B.subtilis*and *K. oxytoca* was 64.3% and 57.1% respectively. Drop dispersion was 33mm with *B.subtilis* and 28mm with *K.oxytoca*. The emulsification rate when using *B.subtilis* and *K.oxytoca* was 78.4% and 59.2% respectively. Our research has prospects to be applied both for microbial enhanced oil recovery (MEOR) and for bioremediation.

Keywords: Hydrocarbon degrading bacteria, biosurfactant, soil pollution,extremophilicbacteries, MEOR

1. Introduction

Extremophilicbacteries are organisms that grow and may even require physically o chemically extreme conditions that are detrimental to the majority of life to develop well. Some extremophiles can adapted simultaneously to live in more than one stress for example Halophile -optimal growth in high concentrations of salt- and Psychrophile-optimal growth under 15ºC and minimal growth at or below 0ºC-(Cavicchioli*et al*., 2000).

These kinds of organisms are of biochemical and biotechnological interest, as they produce extremoenzymes and other metabolites that remain active under severe physico-chemical conditions.

Surfactants have been widely used in the oil industry. Its use spans from the drilling stage until crude oil refining, and can sometimes include the cleaning of storage tanks (Perfumo*et al.,* 2006). For example, it is possible to cite the drilling muds, in which typical composition surfactants are included; these agents act as emulsifiers, dispersants, foaming or anti-foaming agents, and inhibitors of corrosion. In heterogeneous reservoir (Gharbi*et al.,* 1997), stimulation is also crucial the use of surfactants as thin particles dispersant – alkyl phenols and ethoxylated alkyl ammonia – and as corrosion inhibitors – alkyl pyridinium. Furthermore cationic surfactant agents are widely used – fatty amines, imidazolines and quaternary Alkylammonium– as corrosion inhibitors. Within the refined petroleum derivative products assortment is it extensive the use of surfactants. These compounds are added to gasoline and diesel oils for their detergent, dispersant proprieties and as corrosion inhibitors. Lubricant oils for engines contain detergents as petroleum sulfonates, alkyl phenolates or alkyl salicilates of divalent metals (Ba2+, Ca2+, Mg2+). Multi-grade motor oil are surfactant-gelified lubricant oils as lithium soaps; they are used as well sodium, calcium and aluminum soaps or urea polymers (Salager, 1991).

A chemical matter is considered to be surfactant when it causes a significant fall in tension in an interface due to being absorbed in it (Al-Araji*et al.,* 2007). These molecules are amphiphilics, it therefore can be said to posses two different portions.

One of them is the so called polar head, part of the molecule with hydrophilic character and the other is the hydrophobic tale, also known as non-polar portion.Surfactants are produced by way of chemical or biological processes(Hallman*et al.,* 2012).

Extracellular biosurfactants (BS) are involved in cellular adherence, emulsion, dispersion, flocculation, cellular aggregation and desorption phenomena. Although the type and quantity of microbial surfactant depends mainly of the producing organism, factors as carbon source, nitrogen, trace elements, temperature and aeration, such factors have an adverse effect on its production by the organism (Tugruland Cansunar, 2005).

Microorganisms use a wide variety of organic compounds as carbon sources and energy for its growth. When these substrates are inaccessible due to their low solubility and competition with other microorganisms or another type of ambient factor, they use surfactants to get a source of carbon (Medina-Moreno *et al.,* 2011). AlsoBS solubilize insoluble compounds – as hydrocarbons, lipids, oils, pharmacological compounds and others – in aqueous phases: adherence and release of cells in surfaces or biofilm formation, facilitates the transport of hydrocarbons at the membrane level(Haghighat*et al.,* 2008).

Biotechnology advances in large-scale production of metabolites, GMO- genetically modified organisms- as well as the increasing need to protect the environment, provides a promissory future to seriously consider BS as alternatives due its diversity, environmentally friendly, low toxicity and the possibility of production through fermentation processes ex situ/in situ nature and their potential in health care, food industry and of course multipleapplications in the oil industry.

Increasing environmental concern and the emergence of stringent laws have led to BS being a potential alternative(Kaur Sekhon, 2012).

2. Methods and Materials

2.1 Sampling site

A sample of soil polluted with crude oil was taken from an oil field in Surgut, in the region of Siberia–Russian federation (figure 1). The sample was taken at a depth of 5-7 cm, with the aid of a sterile spade which permits getting rid of any excess of humidity from the sample.

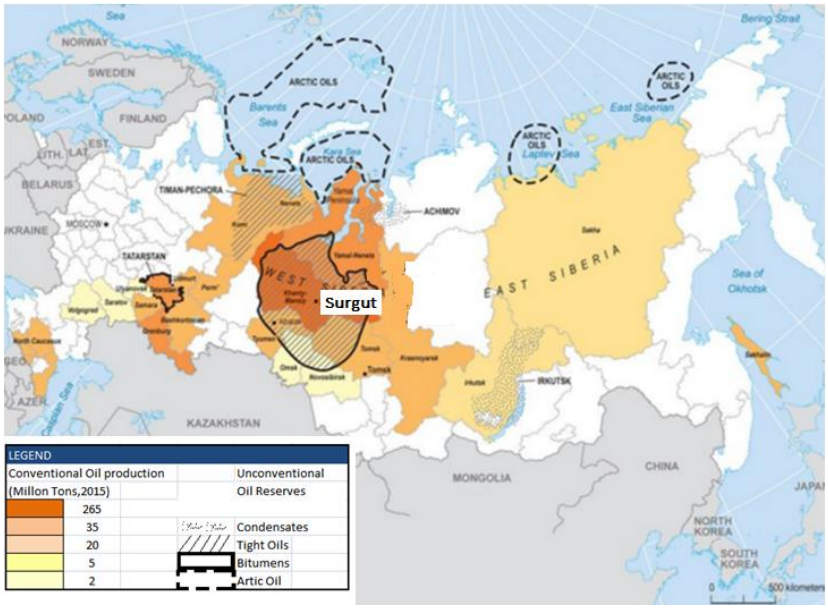

Fig.1Geographical location of Surgut, Siberia region-Russian Federation, coordinates 61°21′ N, 73°26′ E.

2.2 Microbiological isolation

Munz culture media, is a selective culture medium for Oil bacterial strains, the media composition –mass percentaje- is: 0.4 KNO3; MgSO4 • 7H2O 0.08; NaCl 0.1; K2HPO4 0.14; KH2PO4 0.06; Distilled water to fill until 100%, pH 7.2. In a sterile glass flask we add 10 grams of soil sample and poor 90 ml of sterile water.Then we stirred it for 60 minutes, after that 5 ml of the obtained solution was taken and added to a erlenmeryer with 45 ml of Munz liquid media, which contained as sole carbon source 2% crude oil (15 degrees API).They were incubated at 25 ° C , 110 rpm for 15 days (triplicate).After the incubation time were taken 5ml from each erlenmeryer and sowing was repeated once

in liquid medium. This process is repeated for 2 months up to a total of four repetitions ensure stability of the bacterial culture.Met the total period of incubation, the cultures were inoculating on petri Munz agar plate and naphthalene (polycyclic aromatic hydrocarbon, HAP) as sole carbon source, in order to obtain pure colonies. 10^{-2},10^{-4},10^{-6} dilutions were performed, and incubated for 48 hours.

2.3 Removing duplicate colony forming units (phenotypic clones)

After isolation of colony forming units (CFU) morphologically distinct, we proceeded to eliminate clones through MALDI mass spectrometer.MALDI-TOF MS was performed on isolated colonies, which were deposited in duplicate on a test card (96 MSP target ground / polished steel, Bruker Daltonics, Germany GmbH), and proceed to dry at room temperature. Then, the wells were coated with 1 ul of matrix -saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics) in 50% acetonitrile and 2.5% trifluoroacetic acid-.Microflex LT equipment and FlexControl software (version 3.0 Bruker Daltonics) was used. The parameters were adjusted as manufacturer's recommendations. The spectra were analyzed by MALDI Biotyper RTC 3.0 (Bruker Daltonics®, Bremen, Germany) software. The resultstell us how many strains we have without duplicates.

2.4. Screening of biosurfactant- producing bacteria.

The CFU obtained were inoculated in Munzliquid medium with 2% naphthalene and incubated for 7 days. To analyze the biosurfactant production, four methods were evaluated: surface tension measurement, hemolytic activity assay, drop dispersion and emulsification assay.

2.4.1 Surface tension measurement in relation to the growphases

During the incubation period, samples were taken daily (4 ml of every flask) and were centrifuged at 6000 RPM for 15 minutes.The growth phase can be evidenced by turbidity, because of that 4ml of supernatant were taken, using 1.5 mL to measure the optical density at 600 nm (OD 600 Nm) in the spectrophotometer Ekros PE-5300VIL. The remaining 2.5 ml of supernatant was used for measuring surface tension by KRUSS Drop DSA100 shaper analyzer. After 7 days we used 3 complementary assays to check the production of biosurfactant: hemolytic assay, drop dispersion and emulsification measurement.

2.4.2 Blood agar hemolytic assay

Blood agar hemolysis method is used to screen biosurfactant producing strains. This method is based on the fact that biosufactants are able to hemolyse the red blood cell present in blood. This method was developed by Mulligan *et al.*in1985(Naziya*etal.,* 2014).Cultures of selected isolates were spot inoculated on blood agar plates. These plates were

incubated for 24- 48 hours at 37^0C. After incubation plates were observe for zone of hemolysis. This zone of hemolysis indicates production of biosurfactant.

2.4.3 Drop Shape Analysis

The drop shape analysis is another optical method to monitor bacterial biosurfactant production. For screening purposes it was first applied by Van derVegtin 1991 (Rodrigues *et al.,* 2004). The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension. Droplets of liquids with a low surface tension are more apt to deviate from a perfectly spherical shape than droplets of liquids with a high surface tension. For the drop shape analysis, a 100 µl droplet of a bacterial suspension is put on a FEP-Teflon surface. The profile of the droplet is determined with a contour monitor as a function of time up to 2 hours. For this assay, just small amounts of sample are needed. If biosurfactant is present in the cell free culture brothdiameter of this clearing zone indicates the surfactant activity, also called displacement activity. A negative control was maintained with distilled water (without surfactant), in which no displacement or clear zone was observed.

2.4.4 Emulsification assay

Emulsification assay is an indirect method used to screen biosurfactantproduction. Cell free culture broth was used as the biosurfactant source to checkthe emulsification of crude oil. 2 ml of cell free culture broth was addedto 3 ml of mineral oil in a 30 ml screw-capped test tube.The above solution wasvortex-shaken for 2 minute and the emulsion mixture was allowed tostand for 24 hours at 30 °C. A negative control was maintained only with buffer solution and mineral oil was used as the negative control. To determine the rate of emulsification (EI) the following equation was used (Bach*et al*., 2003):

$$EI = \frac{\text{HEIGHT OF EMULSION}}{HEIGHTOFTOTALSOLUTION} * 100\%$$

2.4 Molecular identification of the isolates

After the preliminary microbial isolation and repetitiondiscarding (clones of the same bacteria)the CFU Isolated was send to the MALDI target in duplicate. The method used was suspension of 1 µL loopful (one of the three colonies) of overnight bacterial culture of each isolate in an Eppendorf tube containing 10 µL of 70% formic acid and vortexes to obtain a uniform suspension (i.e, extraction mixture). The extraction mixture was incubated for 30 minutes at room temperature, and then 1 µL of the mixture was spotted onto a MALDI MSP target plate.After air drying for 5 min to allow the formic acid to evaporate, each spot was overlaid with 2 µL ofBiotyper matrix solution and then dried under a

fume hood at room temperature for 10 min. Once dry, the samples were ready for mass spectrometry analysis (GenBank Home, 2016).

The Biotyper was used according to the manufacturer's instructions (Bruker Daltonics). For each sample analyzed, extraction mixtures were spotted in duplicate. The raw spectra were recorded in the positive linear mode within a mass range of 2-20 kDa. Each run was internally calibrated using E. coli ribosomal proteins as a positive control (Bruker Daltonics) and compared to the Bruker Biotyper version 3.0 database(including 5600 MSP- main spectrum profile-) The results of pattern-matching were expressed as a score ranging from 0 to 3 according to the criteria suggested by the manufacturer, scores ≥ 2.3. (2.3-3.0) provide secure species identification; from 2.00 to 2.29 indicate secure genus identification and probable species identification and probable species identification; scores from 1.70 to 1.99 indicate probable genus identification and scores bellow 1.7 indicate that the identification was not reliable. The scores were recorded for each of the duplicate spots and the highest raw score was used for analysis.

3. Results

3.1 Microbiological Isolation

From a sample of soil contaminated from the oil field of Surgut, they were isolated 10 CFU phenotypically different. After removing clones by the mass spectrometer, it was determined that the currently strains obtained were 4, which were identified as *Bacillus subtilis, Bacillus pumilus, Raoultellaornithinolytica and Klebsiellaoxytoca*. Our bacteriesgrowed well in Munz media + crude oil of 15 degrees API as well in Munz media + naphthalene (PAH) as sole carbon sources.The specific gravity scale developed by the American Petroleum Institute (API) refers to the specific gravity of oil as determined density relative to water at 60 ° Fahrenheit.According to this scale our 15 degrees API is considered heavy oil, that's mean denseand viscous. Furthermore, naphthalene is a polycyclic aromatic hydrocarbon, because of that he has not heteroatom or carry substituents.A naphthalene molecule can be viewed as the fusion of a pair of benzene rings with formula $C_{10}H_8$. It can be found naturally in crude oil composition.

Normally, the aromatic compounds are more difficult to degrade, compared to aliphatic hydrocarbons, due to the greater stability of the bonds between carbons present in its structure. Degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons usually decreaseswith increasing carbon number as well of the quantity of aromatic rings (Prince, 2005). Despite the high stability of the petroleum hydrocarbon compounds, the ability to degrade aromatic rings has been described in a variety of microorganisms, including bacterial genera such as *Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Flavobacterium, Mycobacterium, Nocardia, Pseudomonas,*

Rhodococcus, *Sphingomonas*,among others. Isolate crude oil strains andPAH degrading bacteriastrains is very important to make a successful bioremediation strategy or Microbial Enhanced Oil Recovery (MEOR). In addition, the specific conditions of our sampling site suggest that the strains isolated in this study are adapted to extreme conditions such as low temperatures and the presence of recalcitrant compounds, which give it greater potential for application in areas with similar characteristics.

3.2 Biosurfactant production

3.2.1 Surface tension

Substances with surfactant properties have a direct effect on the tension between two surface areas. Therefore the direct measurement of the surface tension is an indicator of the presence of surfactant agents in a sample (Bodourand Miller-Maier, 1998).

As shows in Figure 2 *Bacillus subtilis* and *Klebsiellaoxytoca* showed greater decrease in surface tension (ST) after 7 days (Stationary phase) obtaining a minimum ST of 25 mN/m for the cultivation with*Bacillus subtilis* and 30 mN / m for *Klebsiellaoxytoca*.*Bacillus pumilus* and *Raoultellaornithinolytica* reached 49.9 mN / m and 53.5mN / mrespectively.

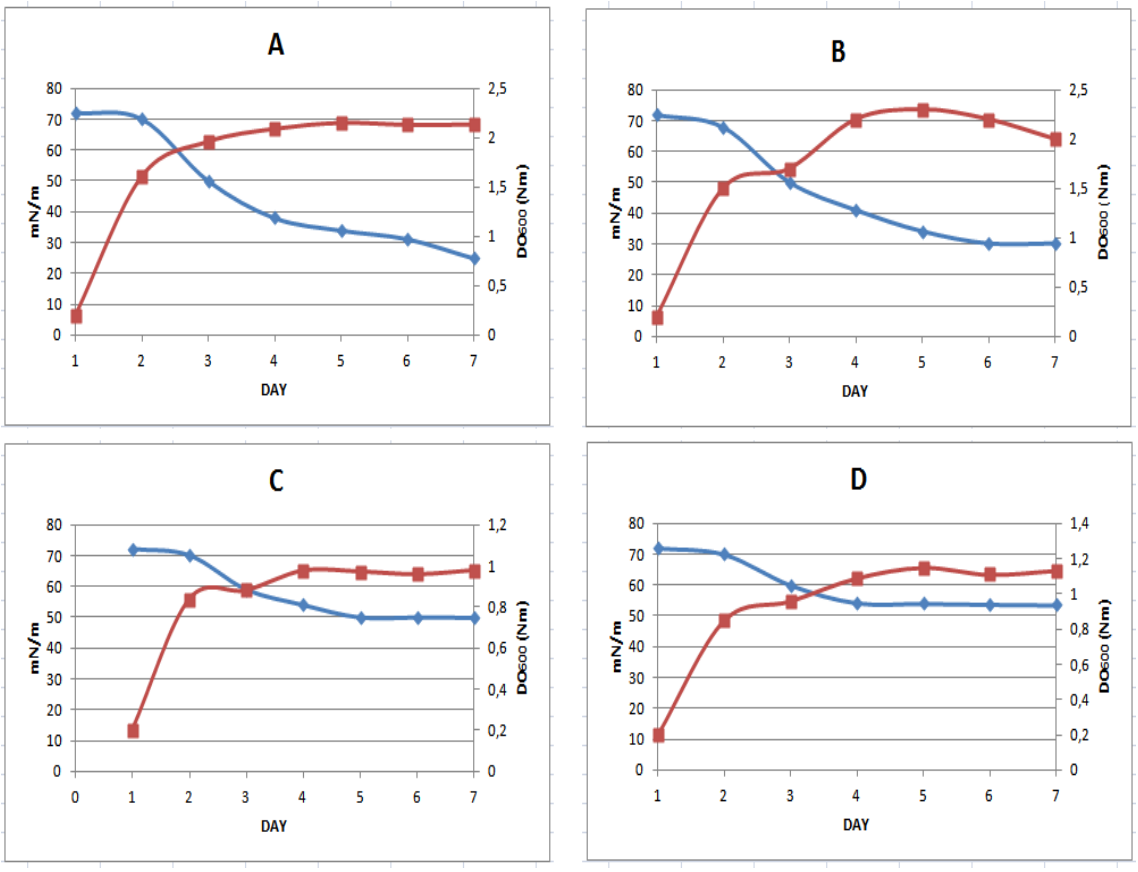

Fig.2Surface tension measurement (ST: mN/m) and growth pattern (DO_{600}Nm) in Munz media by: a)*B. subtilis b)K. oxitoca c)B. pumilus d)R. ornithinolytica*

3.2.2 Blood agar hemolytic assay, drop shape analysis and emulsification assay and surface tension measurement.

All the bacterial strains were screened to check their biosurfactant production with Blood agar hemolytic assay, drop shape analysis and emulsification assay and surface tension measurement. The results (Table 1) show a direct correlation between drop shape analysis and emulsification activity. Strains highly active in any one of these methods were active in the other methods. Similar direct correlation was reported by Youssef Noah (Noha H. Youssef et al.,2004)

Strains *Bacillus subtilis* and *Klebsiellaoxytoca*outcompeted and dominated the other two isolates in all biosurfactant screening studies, involving batch cultures inoculated with equal cell densities and general conditions.

Blood agar hemolysis is a widely used method to screen biosurfactant production due to the simplicity of its realization. In our case it was found that *K. oxytoca*evidenced an absence of haemolytic halos surrounding the colonies, but evidenced decrease in superficial tension and increasing the diameter in drop shape analysis, also has a high emulsificationindex.

We can say that hemolytic assay on blood agar plate is not definitive evidence to judge the biosurfactant production, Authors like Thavasi (Rengathavasi et al., 2013) indicate that hemolytic assay on blood agar plate are not so reliable and sensitive becausethere are other products such as virulence factors thatcan lyse the blood cells and also biosurfactants with poor diffusion agar may not be able to lyse the blood cells.

A strain can be listed as potentially surfactantproducer if shows at leasttwo or three positive results under biosurfactant screening assays.Above earlier reports and results from this studyindicated that tension superficial and oil spreading assays are easy, reliableand sensitive methods to check the biosurfactant production(Afshar*et al.*, 2008).

Tabla-1. Screening of biosurfactant producers.

Name of bacteria	Hemolyticassay	Drop dispersion (mm)	Emulsification index (ei %)
Bacillus subtilis	**+**	**33±0.1**	**78.38**
Bacillus pumilus	+	22±0.1	50
Klebsiella oxytoca	**-**	**28±0.1**	**59.2**
Raoultella ornithinolytica	+	18±0.2	50

Bacillus subtilis and *Klebsiellaoxytoca* can be considered as potential producers of biosurfactants microorganisms. It is well known that *Bacillus subtilis* produces amphipathic molecules that generate foam and reduce surface tension between polar and non-polar molecules, because of that is used inpetroleum recovery and enhanced remediation (Haddad *et al.*, 2009).

About *Klesiella* we found no too much information related to biosurfactants, but*Klebsiellaozaenae*and *Klebsiellaornithinolytica*has been studied as BS producers. (Silva *et al.*, 2014; "Biodegradation - Engineering and Technology," 2013).

By the other hand, *Kleibsella*gender is closely phylogenetically related with enterobacteria such as *Serratia*, which has several species recognized as producers of BS (Alves *et al.*, 2014).

Although *K. oxytoca* strains and *R. ornithinolytica* are considered pathogenic grade 2 on the rating scale groups of bacteria in EU risk (TRBA, 2016), having been isolated from an environmental sample is expected to have no active or lost pathogen genetic traits.

Discussion

Our work has application prospects for enhanced oil recovery and bioremediation. We successfully isolated biosurfactat producing strains and which in turn are degrading and PAH and heavy oil. We recommend the isolated bacteries as they generate BS that can be tested in the cleaning of storage tanks, decrease of the viscosity of heavy oil, increase in the flow inside the pipelines, stabilizers of fuel-water-oil at an emulsions level and also in the regeneration of contaminated soils.

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