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A non-toxic microbial surfactant from *Marinobacter hydrocarbonoclasticus* SdK644 for crude oil solubilization enhancement

Billal Zenati[a,b,*], Alif Chebbi[c], Abdelmalek Badis[a], Kamel Eddouaouda[a], Hocine Boutoumi[a], Mohamed El Hattab[a], Dorra Hentati[c], Manel Chelbi[c], Sami Sayadi[c], Mohamed Chamkha[c], Andrea Franzetti[d]

[a] *Laboratory of Natural Products Chemistry and Biomolecules (LNPC-BioM), University of Blida, 1, Road of Soumaâ, PO Box 270, 09000 Blida, Algeria*
[b] *National Centre for Research and Development of Fisheries and Aquaculture (CNRDPA), 11, Bd Amirouche PO Box 67, Bou Ismail 42415, Tipaza, Algeria*
[c] *Laboratory of Environmental Bioprocesses (LEBP), LMI COSYS-Med, Centre of Biotechnology of Sfax (CBS), University of Sfax, Road of Sidi Mansour Km 6, PO Box 1177, Sfax 3018, Tunisia*
[d] *Department of Environmental Sciences, University of Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy*

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ABSTRACT

This study aims to investigate the ability of a biosurfactant produced by *Marinobacter hydrocarbonoclasticus* strain SdK644 isolated from hydrocarbon contaminated sediment to enhance the solubilization rate of crude oil contaminated seawater. Phylogenetic analysis shows that strain SdK644 was very closely related to *M. hydrocarbonoclasticus* with 16S rRNA gene sequence similarity of 97.44%. Using waste frying oil as inducer carbon source, the producing biosurfactant by strain SdK644 was applied to improve crude oil solubilization in seawater. The preliminary characterization of the produced biosurfactant by FT-IR analysis indicates its possible classification in a glycolipids group. Results from crude oil solubilization assay showed that SdK644 strain biosurfactant was 2-fold greater than Tween 80 surfactant in crude oil solubilization and 12-fold higher than seawater control, as shown by GC-MS analysis of aliphatic compounds. Furthermore, this bioactive compound was shown to be nontoxic against *Artemia* larvae in short-term acute toxicity bioassay. Generally, the results showed the possible use of *M. hydrocarbonoclasticus* strain SdK644 biosurfactant in bioremediation processes of the marine environments.

1. Introduction

Petroleum pollution of the environment is of grave risk because petroleum hydrocarbons are toxic to all forms of life. The contamination of the environment by crude oil is quite common because of its widespread use and its accompanying disposal operations and accidental spills (Zahed et al., 2010). The total input of petroleum hydrocarbons into the oceans from all sources is about 1.300.000 t per year. Alone, natural seeps account for 46% and 37% by all activities associated with consumption of petroleum products. Adding to all this, during transportation of petroleum products, accidental spills and operational discharges of cargo oil contributes with 12% of the total flux discharged, followed by far by extraction processes (3%) (NRC, 2003).

Crude oil -a heterogeneous mixture of hydrocarbons- consists mainly of alkanes, cycloalkanes, and aromatics. Low amounts of resins (nitrogen, sulfur, and oxygen compounds), and asphaltic fraction (partially oxygenated and highly condensed) exist also in crude oil with varying rates depending on the nature of the oil, light or heavy (Tyagi et al., 2011; Weng et al., 2015). Microorganisms capable of degrading hydrocarbons have a ubiquitous existence (NRC, 2003; Vandecasteele, 2005; McGenity et al., 2012). The principal-hydrocarbon degrader bacteria in marine environments are: *Alcanivorax*, *Marinobacter*, *Thallassolituus*, *Cycloclasticus*, *Oleispira,* and a few others (Yakimov et al., 2007; Acosta-González and Marqués, 2016).

The biodegradation kinetics of crude oil in seawater is controlled by numerous factors such as crude oil composition and concentration, temperature, oxygen, nutrients supply, salinity, pH, and oil availability to microorganisms (Vandecasteele, 2005). Petroleum hydrocarbons have a limited bioavailability because they are mostly insoluble in water (Chen et al., 2013). To overcome this kinetic limitation, the application of synthetic surfactants or biosurfactants can promote this availability by reducing interfacial tensions between the two immiscible phases, which leads to increase the surface area of oil slick and therefore improve the solubility of hydrocarbons (Urum and Pekdemir,

* Corresponding author at: Laboratory of Natural Products Chemistry and Biomolecules (LNPC-BioM), University of Blida, 1, Road of Soumaâ, PO Box 270, 09000 Blida, Algeria.
E-mail addresses: bilalzenati@univ-blida.dz, bilal.zenati@gmail.com (B. Zenati).

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2004; Collina et al., 2007; Pacwa-Plociniczak et al., 2011).

The use of biosurfactants is more advantageous over their synthetic counterparts due to their low toxicity, high biodegradability, and effectiveness at extreme temperature, pH (acid/alkaline), and salinity (NaCl) (Desai and Banat, 1997; Roy et al., 2015). Biosurfactants are amphiphilic molecules, with two opposite regions (hydrophilic and hydrophobic), mainly produced by microorganisms such as bacteria, yeast and fungi (Marchant and Banat, 2012a; Healy et al., 1996). Biosurfactants can be classified as lipopeptides and lipoproteins, glycolipids, phospholipids, and polymeric, based on their chemical structure diversity and the type of the producing microorganisms (Healy et al., 1996). The main drawbacks of the large-scale production of biosurfactants are low yields and high production costs (Marchant and Banat, 2012b). Instead, the use of cheap substrates decreases for 10–30% of the total production cost (Mukherjee et al., 2006). Waste frying oil is one of interesting renewable sources employed to synthesis biosurfactants from bacteria and yeasts, with satisfactory production yields achieved (Haba et al., 2000; Zhu et al., 2007; Dzięgielewska and Adamczak, 2013; Li et al., 2016).

Accordingly, this study focused on the isolation of marine hydrocarbonoclastic bacteria with biosurfactant production capacities. The produced biosurfactant by the selected strain was applied to enhance crude oil solubilization from contaminated seawater.

2. Materials and methods

2.1. Chemicals

All chemicals and substrates used in this study were of an analytical grade and were obtained from various commercial sources (BIOCHEM, Panreac, VWR, and Sigma-Aldrich).

2.2. Culture media and conditions

Different media were prepared for isolation and screening of hydrocarbonoclastic microorganisms and for the production of biosurfactants. Their composition was indicated as follows: NSW medium: natural seawater, sterilized by autoclaving at 120 °C for 20 min; and Ch-NSW-CO medium: 4 g chitin, agar 18 g, 1 l NSW. The medium was poured into petri dishes and allowed to solidify. After that, 1 ml of sterile crude oil (CO) (autoclaved for 30 min at 120 °C) was sprayed on the surface of this medium; Ch-NSW medium: 4 g chitin, agar 18 g, 1 l NSW was also sterilized; NSW-CO medium: 18 g agar, 1 l NSW with 1 ml CO; and Luria Bertani (LB): 10 g peptone, 5 g yeast extract, 23 g NaCl in 1-litre distilled water. The pH was adjusted to 7 ± 0.2 with a solution of 1 M caustic soda (NaOH). For the preparation of a solid LB, 18 g agar was added to the medium; Mineral salt medium (MSM) (g l^{-1} distilled water): 23 NaCl, 0.4 NH_4Cl, 0.3 KH_2PO_4, 0.3 K_2HPO_4, 0.33 $MgCl_2$, 0.05 $CaCl_2$, and 1 ml of trace metals containing (mg l^{-1} distilled water): 1500 $FeCl_2{\cdot}4H_2O$, $CoCl_2{\cdot}6H_2O$ 190, 100 $MnCl_2{\cdot}6H_2O$, 70 $ZnCl_2$, $6H_3BO_3$, 36 $Ma_2MoO_4{\cdot}2H_2O$, and 6.7 ml HCl 35%. The media were autoclaved at 120 °C for 20 min.

2.3. Isolation of marine bacteria

Marine bacteria were isolated from fishing harbor severely contaminated by petroleum products (diesel and waste lubricant oil) located in Bou-Ismail bay, Khemisti Fish Harbor (W. Tipaza, Algeria). For isolation procedure, 5 g of superficial sterile sediment was inoculated in 100 ml NSW-CO medium. Enrichment cultures were incubated at 30 °C on a rotatory shaker at 150 tr min^{-1} for 7 days. After three consecutive subculture (3 ml of aliquot) in fresh NSW-CO medium, the suspension was serially diluted and plated onto Ch-NSW-CO medium. Purified isolates were obtained by streaking colonies several times in solid LB and NSW-CO media and stored at – 80 °C in LB broth supplemented with glycerol (0.25, v/v).

2.4. Screening of isolates capable of growing on crude oil and producing biosurfactants

A collection of sixteen bacterial isolates were screened for their ability to grow on crude oil as the sole carbon and energy source. Regarding on its high growth rate on crude oil, the SdK644 strain was selected in this study. These strains were routinely cultured and maintained in the LB medium at 30 °C and 150 tr min^{-1}. Cultivations were performed in 250 ml flasks containing 100 ml MSM and CO (1%, v/v). Kinetic growths were monitored by measuring optical density (OD) at 600 nm. To avoid the false values of OD due to hydrocarbon particles absorption, each culture sample (2 ml) was centrifuged in Eppendorf tube at 8050 × *g* for 10 min. The pellet was then resuspended in 2 ml sterile physiological solution (9 g NaCl per litre of distilled water), vortexed at high speed for 2 min and OD_{600} of suspension was therefore measured. Systematically, the production of biosurfactants, as agents facilitating the access of hydrocarbon to the microorganisms was detected by measuring the surface tension (ST) of culture supernatants as previously reported (Eddouaouda et al., 2012).

2.5. 16S rRNA sequencing and phylogenetic analysis

The 16S rRNA gene of the strain SdK644 was amplified by PCR using a Stratagene PCR system (Robocycler gradient 96) with GoTaq DNA polymerase (Promega, Madison, WI, USA). The universal primers Fd1 and Rd1 (Fd1, 5′ AGAGTTTGATCCTGGCTCAG-3′; Rd1, 5′-AAGG AGG-TGATCCAGCC-3′) were used to obtain a PCR product of approximately 1.5 kb. Positions of sequence and alignment ambiguity were omitted, and pairwise evolutionary distances based on 969 bp unambiguous nucleotides, were calculated using the method of Jukes and Cantor (1969). A dendrogram was constructed using the neighbour-joining method (Saitou and Nei, 1987) with the Molecular Evolutionary Genetic Analysis (MEGA) program version 6 (Tamura et al., 2011).

The 16S rRNA gene sequence of strain SdK644 was determined and deposited in the GenBank/DDBJ/EMBL nucleotide databases under accession number KU680815.

2.6. Biosurfactant application for enhanced crude oil solubilization

2.6.1. Biosurfactant production

Applied biosurfactant was produced by the bacterial strain SdK644 in MSM supplemented with yeast extract (0.01%, w/v). Waste frying oil was used as inducing carbon source, NH_4Cl as nitrogen source, C/N of 50 (g of waste frying oil/g of NH_4Cl), a pH medium of 7, a salinity (NaCl) of 23 g l^{-1} and an optimal cultivation time of 3 days.

The medium was distributed into 5-liter Erlenmeyer flask with a working volume of 2-liter. The medium was inoculated at 2% (v/v) with an overnight culture of strain SdK644 maintained in LB broth and incubated at 30 °C and 150 tr min^{-1}.

The crude biosurfactant was recovered according to the protocol described by Saikia et al. (2012) with slight modification. Briefly, the culture supernatant was extracted twice with ethyl acetate, concentrated under vacuum and the crude biosurfactant (yellow viscous liquid) was dried and weighed.

2.6.2. Partial characterization of biosurfactant

A biosurfactant stock solution was prepared and diluted to obtain different concentrations (3 − 6300 mg l^{-1}). For each solution, the ST was measured as previously described (Eddouaouda et al., 2012). The critical micelle concentration (CMC) of the biosurfactant was also determined.

The crude biosurfactant from strain SdK644 was analyzed in Bruker-TENSOR 27 Fourier transform infrared spectroscopy (FTIR)-Spectrometer. The infrared absorption spectrum was measured at the region bonds of 400 − 4000 cm^{-1}. One drop of crude biosurfactant was placed in the middle of KBr pellet with 1–2 mm thickness. The KBr