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Exploring the potential of halophilic bacteria from oil terminal environments for biosurfactant production and hydrocarbon degradation under high-salinity conditions

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ABSTRACT

The wastewater from oil production can be exceptionally saline and contain a complex mixture of hydrocarbons, many of which are highly toxic. This study aimed to identify and characterize 141 halophilic bacteria isolated from production water and activated sludge from Marine Terminal Almirante Barroso (Brazil) and evaluate their potential for biosurfactant production and biodegradation of distinct petroleum hydrocarbons. Sequencing and phylogenetic analysis of the 16S rRNA gene revealed that the halophilic bacteria retrieved are distributed among 20 genera and four phyla (Proteobacteria, Firmicutes, Actinobacteria and Flavobacteria). RAPD fingerprinting was used to differentiate isolates at the infraspecific level, revealing 79 different genetic profiles. GC-MS analysis carried out with eight strains confirmed their ability to efficiently degrade alkanes and aromatic compounds under halophilic conditions, with preference for aromatic degradation. Eleven strains showed significant ability for reduction of the surface tension (from 72 to 40 mN/m) and for emulsification (up to 71%) of four different types of oils (mineral, soybean, diesel and kerosene). Results gathered in this study demonstrate a high taxonomic and genetic diversity of the halophilic bacterial strains isolated from the oil terminal samples and an outstanding potential for further use in biotechnological processes such as biosurfactant production or bioremediation.

1. Introduction

Oil is a complex mixture of organic and inorganic compounds and has as its most important constituent hydrocarbons, which can reach up to 98% of the total composition (Bícego, 1988). Oil composition varies significantly depending on the source reservoir and may contain sulfur, nitrogen, oxygen and metal compounds (Freedman et al., 1995; Marques et al., 2009). Polycyclic aromatic hydrocarbons (PAHs) found in oil can enter the environment through human and natural activities and are considered as environmental contaminants (Johnsen et al., 2005). PAHs are an increasing concern because of their toxic, mutagenic, and carcinogenic properties (Tang et al., 2005).

Biodegradation of hydrocarbons is a widely known metabolic process that has been reported for many bacterial genera (Seo et al., 2009), including *Halomonas* (Wang et al., 2007), *Alcanivorax* (Yakimov et al., 1998), *Marinobacter* (Gauthier et al., 1992), *Dietzia* (Borzenkov et al., 2006), *Bacillus* (Kumar et al., 2007; Sass et al., 2008), *Oleiphilus* (Golyshin et al., 2002), *Oleispira* (Yakimov et al.,

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2003) and *Geobacillus* (Chamkha et al., 2008), among others. The technology commonly used for hydrocarbon remediation includes mechanical, burying, evaporation, dispersion and washing procedures. The mechanisms employed for hydrocarbon removal from the environment rely on expensive, slow and inefficient methodologies (Mandri and Lin, 2007). Biological treatment or bioremediation is a desirable alternative due to its low cost and high efficiency (Stallwood et al., 2005; Karhu et al., 2009). Bioremediation uses microorganisms to degrade toxic pollutants and convert them into less toxic or harmless products, offering an environmentally safe and cost-effective technique (Kumar et al., 2011).

In order to enhance the solubility and bioavailability of these hydrophobic compounds, many bacteria produce biosurfactants, thus facilitating their uptake and biodegradation (Bodour and Maier, 2002). Biosurfactants are distributed into various categories such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Cappello et al., 2012). The biosurfactants are excellent agents for emulsification, detergency, dispersion, microbial growth enhancement and metal sequestering (Pacwa-Plociniczak et al., 2011). These important characteristics make them suitable for use in oil recovery and bioremediation, with great potential for future replacement of the chemical surfactants (Ghanavati et al., 2008).

Information on microorganisms involved with PAH biodegradation in moderate to high salinity environments in the last two decades is extensive. There is, however, little information on the ability of biosurfactant production in highly saline environments. In the present study, isolation, taxonomic identification and genetic characterization of halophilic bacteria from production water and activated sludge originated from a petroleum terminal were performed giving further assessment of their potential to biodegrade PAHs and produce biosurfactants under high salinity conditions.

2. Material and methods

2.1. Sampling, acclimation and bacterial isolation procedure

The bacterial strains used in the present work were isolated from activated sludge of wastewater treatment and production water from Marine Terminal Almirante Barroso (TEBAR, São Sebastião, SP, Brazil). This site is located in the north coast of São Paulo state and receives oil mixed with high salinity production water originated from offshore platforms. Sampling was performed by the technical staff of PETROBRAS. Two liters of activated sludge and production water were collected using sterilized plastic flasks and stored at 4 °C for transportation to the laboratory in the Federal University of Viçosa (UFV), MG, Brazil. After arrival, bacterial isolation procedures were immediately performed.

Aliquots of 1 mL of sludge and production water were serially diluted (10^{-1} to 10^{-8}) in saline solution and 100 μL of each dilution were inoculated onto Petri dishes containing one of the four culture media: R_2A (Difco) (Reasoner and Geldreich, 1985), MOD (Rohban et al., 2009), SAL (saline) (Pagaling et al., 2009) and MCAT (casamino acid) (Litchfield et al., 2009). Sodium chloride (4%) was added to all culture media in order to mimic the reactor condition to which microorganisms were already adapted to. Petri dishes were incubated at 28 °C and monitored for colony growth.

For sludge acclimation to high salinity condition, the media R_2A (Difco) (Reasoner and Geldreich, 1985) and Moderate (MOD) (Rohban et al., 2009) were employed, since they allowed the recovery of a higher bacterial diversity in the isolation procedure. Five milliliters-aliquots of activated sludge were inoculated into 250 mL-Erlenmeyer flasks containing 50 mL of liquid medium added of 6–20% NaCl. Flasks were incubated in rotational shaker at 28 °C and 150 rpm. Every 6 days, acclimation cultures were transferred to fresh medium containing 1% higher salt concentration, until salt concentration reached 20%. During acclimation process, 100 μL-aliquots of each salt concentration culture were plated on the surface of solid MOD and R_2A media. After growth, colonies with different morphologies were selected and submitted to streaking onto individual Petri dishes and incubation at 28 °C until the appearance of new colonies. Each culture was submitted to stereomicroscope analysis, followed by Gram staining to verify purity of cultures.

2.2. Bacterial identification

Genomic DNA extraction from all bacterial isolates was performed according to the protocol described by Soolinger et al. (1993). 16S rRNA gene was partially amplified from genomic DNA by PCR using the primer set 10f (5′ GAG TTT GAT CCT GGC TCA G 3′) and 1100r (5′ AGG GTT GCG CTC GTT G 3′) (Lane, 1991). PCR was performed in 25 μL-reaction mixtures containing 0.5 μM each primer, 0.2 mM dNTPs (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 2.0 U *Taq* polymerase (Invitrogen) and 1.0x reaction buffer (Invitrogen) and 50–100 ng genomic DNA. Amplification was conducted using an Eppendorf Mastercycler Gradient (Eppendorf Scientific, New York, USA) and the program consisted of 1 cycle for denaturation at 95 °C for 1 min, followed by 30 cycles of 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing, and 3 min at 72 °C for extension, with a final extension step of 3 min at 72 °C.

PCR products were purified using Illustra PCR DNA and Gel Band Purification Kit (GE Healthcare), and used as template for sequencing in the ABI3500XL (Applied Biosystem) sequencer with ABI Genetic analyzer BigDye Terminator cycle sequencing Kit (Applied Biosystems), buffer BigDye Terminator v1.1 (5× sequencing Buffer-Life Technologies) (0.1 M Tris-HCl, 0.5 mM $MgCl_2$) and 3.2 pmol each primer (10f and 1100r).

Partial 16S rRNA gene sequences obtained from isolates were assembled in a contig using the phred/Phrap/CONSED software (Ewing et al., 1998; Gordon et al., 1998). Sequences were compared against sequences of closely related type strains retrieved from the GenBank (http://www.ncbi.nlm.nih.gov) (Benson et al., 2013) database and RDP (Ribosomal Database Project, Wisconsin, USA, http://rdp.cme.msu.edu), using BLASTn and SequenceMatch routines, respectively. Sequences were aligned using the CLUSTAL X program (Thompson et al., 1997) and analysed with MEGA software v 6.06 (Tamura et al., 2013). Phylogenetic reconstruction was done with the neighbor-joining method (Saitou and Nei, 1987) and Kimura's two-parameter model (Kimura, 1980), with bootstrap values calculated from 1000 replicate runs.

2.3. RAPD fingerprinting

The molecular technique RAPD (Random Amplified Polymorphic DNA) was employed in order to genetically differentiate the bacterial isolates belonging to the same species. Three primers of Set 100/1 (University of British Columbia, Vancouver, Canada) were used for typing the bacterial strains in RAPD independent reactions (Burgess et al., 2005), as follows: UBC # 2 (5′ - CCT GGG CTT G – 3′), # 4 (5′- CCT GGG CTG G – 3′) and UBC # 25 (5′ - ACA GGG CTC A - 3′) for Proteobacteria, and UBC # 4 (5′- CCT GGG CTG G – 3′), UBC # 18 (5′- GGG CCG TTT A– 3′) and # 25 (5′ - ACA GGG CTC A - 3′) for Actinobacteria and Flavobacteria. Twenty five microliter-reaction mixtures contained 5 ng of genomic DNA, 2 U of *Taq* DNA polymerase (Invitrogen), 1X *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM of

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