Biodegradation potential of bacterial isolates related to the genus *Thalassospira* in hypersaline conditions

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ABSTRACT

Three bacterial species related to the genus *Thalassospira* (*T. lucentensis*, *T. xianhensis* and *T. profundimaris*), isolated from polluted sediment and seawater samples collected from Priolo Bay (eastern coast of Sicily, Ionian Sea), were analyzed for their biotechnological potential. For this purpose, were evaluate the presence/absence of specific catabolic genes associated to aliphatic and aromatic hydrocarbons metabolism, the production of biosurfactants and emulsification activity, the capability to degrade oil derived linear, branched, cyclic alkanes and polycyclic aromatic hydrocarbons (PAHs). Alkane hydroxylase gene (alkano-monoxygenase *alkb* and citocrome *P450*) and biphenyl dioxygenase gene (*bphA* and *xylC1)* were present in genome of strains, confirming their hydrocarbons degrading capability. All strains of *Thalassospira* produced biosurfactants and showed emulsification activity. The two-dimensional gas chromatography analysis (GC×GC) showed that they were able to degrade oil fractions with the capacity ranged between 77-91%.

Data obtained in this study demonstrated the potential of *Thalassospira* for oil bioremediation in oil-contaminated coastal area, suggesting that the three bacterial strains isolated could be used for bioremediation purposes and treatment of contaminated ecosystems.

Keywords:

Bioremediation, Marine Environment, Marine Oil Pollution, *Thalassospira*, Comprehensive two-dimensional gas chromatography

1. Introduction

Bioremediation consist in the conversion of environmental pollutants in non-toxic compounds by microrganisms. During this transformation, organic and toxic compounds were convert into less complex and toxic metabolites, and with the mineralization process in inorganic compounds, H_2O and CO_2 (if the microorganisms are in aerobic condition) and CH_4 (if the microorganisms are in anaerobic condition) (Nikolopoulou and Kalogeraki, 2010).. The successes of bio-conversion depend on many factors, such as: pH, temperature, oxygen concentration, availability of nutrients and/or chemicals, type of pollutants, cellular transport properties, and chemical partitioning in growth medium (Singh and Ward, 2004). On the foregoing basis, bioremediation techniques have proved advantageous, and the study and the optimization of this eco-friendly technique can be a valid alternative to chemical and physical techniques for cleaning up of oil polluted marine environments (Alkatib et al., 2011). Oil derived hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater and soil habitats (Chaillan et al., 2004; Yakimov et al., 2007,Catania et al., 2015, Catania et al, 2017, Djahnit et al., 2019). Despite the chemical stability, very low water solubility and bioavailability of hydrocarbon compounds, numerous bacterial species have been described as efficient break-downer of oil-pollutant as widely evidenced in literature (Cerniglia 1993; Sutherland et al. 1995; Kanaly and Harayama 2000; Peng et al. 2008; Haritash and Kaushik 2009, Catania et al., 2020a). Scientific interests and biotechnological applications about hydrocarbon-utilizing bacteria increased in recent years (Margesin and Schinner 2001; Le Borgne et al. 2008, Scaffaro et al., 2017, Catania et al., 2020b) and several studies on the biodegradation of crude oil and/or PAHs and other aromatic compounds have been performed (Kasai et al. 2002; Melcher et al. 2002; García et al. 2005; Li and Bai 2005; McKew et al. 2007, Catania et al., 2018). Bacterial degradation of PAHs was firstly described by Zhao et al. (2009), in a phenanthrene degrading consortium from a saline soil which was developed at 10% salinity

and included several strains belonging to different genera, among which *Thalassospira spp*. (Hassanshahian et al., 2010 and 2012). Members of *Thalassospira* genus are mesophilic, halophilic, Gram-negative, oxidase and catalase positive, aerobic and often facultative anaerobic α-Proteobacteria with the ability to utilize hydrocarbons or xenobiotics compound such as polyvinyl-alcohol as source of carbon and energy. (Baldani et al., 2014; Kiseleva et al., 2015). Recent studies revealed and demonstrated widely as the genus *Thalassospira* is correlated to hydrocarbons pollution in marine environment (Lopez Lopez, A. et al 2002, Chen, et al. 2007, Kodoma et. al. 2008, Zhaoet al. 2010, Thompson et al. 2017, Catania et al., 2018,),

Furthermore, numerous scientific evidences showed that some *Thalassospira* species were the dominant members in the hydrocarbon-degrading consortia, suggesting a central role in biodegradation and restoration of marine ecosistems.(Muangchida, et al. 2018).

however, the knowledge on some species of the genus *Thalassospira* is still limited, and the capabilities and mechanisms of degradation of contaminants are not well known.

In this study, for the first time, the biotechnological potential (for bioremediation application) of species. *lucentensis*, *xianhensis* and *profundimaris* related to the genus *Thalassospira* (*T*) were analyzed. In particularwere evaluate: i) presence/absence of specific catabolic genes, ii) production of biosurfactants and iii) capability to degrade different oil fractions (linear, branched, cyclic alkanes and polycyclic aromatic hydrocarbons). The potential of hydrocarbons degradation was evaluate using a comprehensive two-dimensional gas chromatography method (GC×GC) with a dual detection, flame ionization detector (FID) and single quadrupole (Q MS). The use of this instrument has proved essential in order to achieve a detailed characterization and quantification of the different chemical families (Tranchida et al., 2009, Zoccali et al., 2015, 2018).

2. Materials and methods

2.1. Isolation of bacterial strains

Bacterial strains were isolated from enrichment cultures obtained from seawater and sediment samples collected in Priolo Bay (South-East Sicily, Italy, Mediterranean Sea) (Catania et al., 2015). As previously reported (Catania et al., 2015) different enrichment sets were performed by supplementing the ONR7a medium (Dyksterhouse et al., 1995), with n-alkanes mixture (C_{16}, C_{18} and C_{24}), PAHs (phenanthrene, pyrene, biphenyl and dibenzothiophene) or crude oil (Arabian Light Crude Oil). Phenotypically different colonies obtained from the plates were purified, and transferred to fresh medium with hydrocarbons as unique carbon sources (crude oil, n-Alkanes mixture, PAHs). Plate without hydrocarbons was prepared to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil, *n*-alkanes mixture or PAHs were stored in stock media with glycerol at -20±1°C for further characterization (Hassanshahian et al., 2012). On the base of the data present in literature and 16S rDNA analysis, the isolates more interest were selected to following analysis.

2.2. Identification of the isolates

2.2.1. Molecular characterization of isolates (16S rDNA sequencing)

Total DNA extraction of bacterial strains obtained was performed through the MasterPure Complete DNA&RNA Purification Kit (Epicenter, Biotechnologies, Madison, WI) in accordance with manufacture's protocol. The 16S rDNA loci were amplified using the 27F (5'-AGAGTTTGATCCTGGCTCAG-3', Lane, 1991) primer and the 1492R (5' TACGGYTACCTTGTTACGACT-3', Lane, 1991) universal primer. Polymerase chain

reaction (PCR) was carried out in 50 µL of reaction mixture containing 1x reaction buffer, 1x solution Q (both from QIAGEN), 1 µM of each primer, 200 µM dNTP (Gibco), 1 µL of template and 2.5 U of Qiagen *Taq* polymerase. The PCR conditions were as follows: 95 °C for 5 min (1 cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 2 min (35 cycles); with a final extension step at 72°C for 10 min. PCR products were sequenced using Macrogen Service (Macrogen, Europe). The analysis of the sequences was performed as previously described by Yakimov et al. (2005). Sequences similarity of individual inserts was analyzed by the FASTA program Nucleotide Database Query, available through the EMBL-European Bioinformatics Institute. The phylogenetic affiliation of the sequenced strains was performed as described by Yakimov et al. (2006). 16S rDNA were deposited in GeneBank with Accession Number: KT348286, KT348281 and KT348287.

2.2.2. Biochemical identification

In addition to the morphological examination carried out in Catania et al. 2015 was perfomed: i) Oxidase/Catalase test; ii) cellular shape and mobility. These tests done according to the Bergey's manual for identification taxonomy (Holt et al. 1998).

2.2.3. Qualitative analysis of catabolic genes

Qualitative analysis (presence/absence) of catabolic genes [cytochrome (*P450),* alkane hydroxylase (*alkB)* and biphenyl dioxygenase iron sulfur protein (*bphA* gene) were carried out using different couples of primers. The presence/ absence of *P450* gene (~800 bp product) was assayed applying the primers P450F (5′TGTCGGTTGAAATGTTCATYGCNMTGGAYCC-3′) and P450R (5′TGCAGTTCGGCAAGGCGGTTDCCSRYRCAVCKRTG-3′)(Kubota *et al.*, 2005); while to assay the presence/ absence of alkane-monoxygenase gene (~550 bp product)

was used degenerate primers alkBwf (5′-AAYACNGCNCAYGARCTNGGVCAYAA-3′ and alkBwr (5′-GCRTGRTGRTCHGARTGNCGYTG-3’) (Kloos et al., 2006), and couple primers TS2S (5’-AAYAGAGCTCAYGARYTRGGTCAYAAG-3’) and deg1RE (5’-GTGGAATTCGCRTGTCIGARTG-3’) (Smits et al., 1999); the primers described by Ryslava et al. (2003) 352F (5’-TTCACCTGCASCTAYCACGGC -3’) and 1178R (5’-ACCCAGTTYTCDCCRTCGTCCTGC-3’) were used for the detection of *bphA* gene (~800 bp product). The PCR reaction mixtures contained 50 ng of DNA, 1× Qiagen reaction buffer, 1× solution Q (Qiagen), 0.4 mM P450 primers (or 0.8 mM for *alkB* primers and/ or 10 pmol for *bphA* primers), 200μM dNTPs (Gibco) e 2.5 U di Qiagen Taq Polymerase in a final volume of 50 ml; while the PCR reaction mixtures employed with the primers TS2S and deg1RE was carried out as described by Innis et al (1990). The PCR amplification of alkane-monoxygenase gene and P450 gene was carried out on a Mastercycler Gradient (Eppendorf) with the following conditions: initial denaturation 4 min at 94°C (1 cycle); 30 s at 94°C, 30 s at 52°C (for the degenerate primers alkBwf and alkBwr, it was 30 s at 55°C) and 1 min at 72°C (32 cycles); and a final extension at 72°C for 10 min; while for the amplification of alkane-monoxygenase gene with the primers TS2S and deg1RE and *bphA* gene were used respectively the condition described by Smits et al. (1999) (1 cycle 4 min at 95°C; 25 cycles: 45 s at 95°C, 1 min at 40°C, 1 min at 72°C; 1 cycle 5 min at 72°C, indefinitely at 4°C) and Dudášová et al. (2014)(at 95 °C for 2 min; 30 cycles: 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and 30 s; and at 72 °C for 10 min). The gene amplicons obtained with PCR reaction were separated by electrophoresis on agarose (1.5%) gel (Whang et al.2010).

2.4.1. Starter culture and growth in laboratory conditions

The initial cultures were prepared by inoculating one loop of microbial cells into 10 mL of ONR7a mineral medium based on seawater (Dyksterhouse et al., 1995) supplied with 5% of Sodium Acetate. After growing in a rotary shaker (New Brunswick C24KC, Edison NJ, USA;

150 ×g) at 25±1 °C for two days the culture was used to Biosurfactant Production, Emulsification Activity and Liquid Surface Tension, besides 500 µL of the seed-culture broth were washed (×2) with PBS 1× and ready for Biodegradation experiment.

2.3. Biosurfactant Production, Emulsification Activity and Liquid Surface Tension

Biosurfactant production was screened by two distinct methods: *i*) The Drop collapse test and *ii*) Oil Spreading. In the Drop collapse test, 7 µl of (sterile) mineral oil was added to each well of a 48-well microtiter plate. Covered plates were equilibrated for 24 hours at room temperature before adding culture supernatant. 20 µl of the culture supernatant was added on the (sterile) oil surface in the microtiter well. Sodium dodecyl sulfate (SDS, Sigma-Aldrich, Milan) and sterile distilled water were used respectively as positive and negative control suspensions (Manjoubi et al., 2013). In the Oil Spreading, 20 ml of distilled water was added to an empty Petri dish followed by addition of 10 µl of crude oil to the surface of the water. An aliquot of 10 microliters of bacterial culture were then added to the oil surface. The diameter of the clear zone around the bacterial suspension corresponds to the biosurfactant concentration (Youssef et al., 2004). Emulsification activity was measured with the Emulsification index (E_{24}). The E24 was determined by adding 2 ml of hydrocarbon (benzene-C_6H_6 and diesel) to same volume of different bacterial culture. After mixing with a vortex for 2 min and leaving to stand for 24 h, the E_{24} index is given as percentage of height of emulsified layer (in millimeters) divided by total height of the liquid column (in millimeters) (Iqbal et al. 1995; Cappello et al., 2012). The Surface Tension of bacterial cultures was measured after 10 days of growth, by the Wilhelmy plate method, using a digital tensiometer (Gibertini, Italy), in accordance with the manufacturer's instructions. The surface tension was expressed in units of mN m^{-1}.

194 *2.4. Biodegradation experiment*

195 *2.4.2. Experimental set-up of microcosm systems*

196

197 The microcosm systems were performed in 250 mL sterilized Erlenmeyer flasks.
198 Microcosms were incubated at 25±1°C for 15 days with shaking (100 ×*g*) and were realized in
199 100 mL of mineral ONR7a medium supplemented with 0.1% (v/v) -of sterile crude oil (Arabian
200 Light Crude Oil; ENI Technology S.p.A.) and inoculating single bacterial strains. Crude oil
201 used in the experimentation was introduced in microcosm systems after physical weathering
202 (100 ×g, 25±1°C for 48 h), and supplemented with 0.1% (v/v) of heptamethylnonane ($C_{16}H_{34}$,
203 Sigma-Aldrich, Milan) as internal spike for measure of bio-degradation rate. Abiotic
204 microcosms untreated (without inoculation) were used as negative control. All
205 experimentations were carried out in twice.

206

207 *2.4.3. Hydrocarbons extraction of microcosm system*

208 After acidification TERHCs from microcosm system were extracted at room temperature
209 on a shaking table using dichloromethane (CH_2Cl_2, Sigma-Aldrich, Milan, Italy; 10% v/v). This
210 procedure was repeated thrice, and the CH_2Cl_2 phase was combined and treated with sodium
211 sulfate anhydrous (Na_2SO_4, Sigma-Aldrich, Milan, Italy) in order to remove any residual water
212 (Ehrhardt et al., 1991; Wang et al., 1998; Dutta and Harayama, 2001; Denaro et al., 2005). The
213 extracts were concentrated by rotary evaporation (Rotavapor model R110; Büchi Labortechnik
214 AG, Switzerland) at room temperature (~30 °C), followed by evaporation under a stream of
215 nitrogen.

216

217 *2.4.4. Cryogenic Modulation GC×GC-Q MS/FID Analyses*

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Quali-, quantitative analysis of hydrocarbons from microcosm were analyzed by Cryogenic Modulation (CM) GC×GC-Q MS/FID consisting of two GC-2010 gas chromatographs (Shimadzu Corporation, Kyoto, Japan), a TQ8040 triple quadrupole mass spectrometer (Shimadzu) and a flame ionization detector (FID). Protocol use for extraction was following the 3510 EPA (Environmental Protection Agency). After 15 days of incubation sample were collected from microcosms to analyze the residual hydrocarbons. All Data were collected by the GCMS Solution v.4.41 software (Shimadzu); the ChromSquare ver 2.3 software was used for GC × GC data processing (Shimadzu). MS spectral matching was performed automatically by using the ChromSquare ver 2.3 software and the Wiley Registry 11th Edition/NIST 2017 Mass Spectral Library (Wiley, NIST). Moreover, the identification was performed by comparison with linear retention index, and elution order. The first gas chromatograph (GC1) was equipped with an AOC-20i auto-injector, and a split–splitless injector (350°C). The primary column (^{1}D) was an SLB-35 ms [(silphenylene polymer, which can be considered equivalent in polarity to poly(35%diphenyl/65% dimethylsiloxane)] with dimensions 30 m × 0.25 mm id × 0.25 μm d_f. A 1.0 m × 0.10 mm id uncoated column was used to create the modulator loop. The second dimension (^{2}D) column was an SLB-5 ms [(polymer which can be considered equivalent in polarity to poly(5%diphenyl/95% dimethylsiloxane)] with dimensions 1.0 m × 0.10 mm id × 0.10 μm d_f. All the columns used were provided by Merck Life Science (Merck KGaA, Darmstadt, Germany). The connections between the ^{1}D and ^{2}D columns, and the modulator loop, were made by using two SilTite mini unions (Trajan, Ringwood, Victoria, Australia). The outlet of the ^{1}D column was connected to a MXT “Y”-Union (Restek); the latter was then linked to a 0.4 m × 0.10 mm id (for FID analysis) and to a 1.0 m × 0.10 mm id uncoated column (for MS analysis). An average of 63% of the column effluent was directed to the FID with the objective of quantification, while the remaining fraction reached the MS for identification purposes. Helium was supplied at the GC1 inlet at a

pressure of 289.5 kPa (constant average linear velocity mode); volume and mode of injection: 1 μL in the split mode (25:1). GC1 temperature program: 80–350°C (20 min) at 3°C min^{-1}; GC2 temperature program: 60-350 °C (13.3 min) at 3 °C min^{-1}. Modulation was performed by using a cryogenic fluid-free modulator; modulation period was 4 s (the heating step was performed at 400 °C, for 0.35 s). MS conditions: the temperature of the interface was 330°C; the ion source temperature was 280 °C, with analyte fragmentation induced by electron ionization (70 eV). A spectral generation frequency of 33 *Hz* was applied with the following mass range *m/z* 45-360. FID (360 °C) sampling frequency was 125 *Hz*.

3. Results

3.1 Taxonomic and Biochemical characterization of isolates in study

Three different bacterial strains HC-degrading were isolated by sediment and seawater samples in Priolo Bay as previously reported in Catania et al. (2015). The isolates were identified by 16S rDNA analysis and were affiliated to genus *Thalassospira* (Alpha-proteobacteria class), species *T. xianhensis*, *T. lucentensis* and *T. profundimaris*. The results of phylogenetic identification are shown in **Fig.1.** The three bacterial strains formed an independent branch related to other type strains HC degrading affiliated to other genus among Alpha proteobacteria. In particular, The sequences showed considerable evolutionary divergence from the sequences of *Aquaspirillum* sp., *Terasakiella* sp.,. Colonies were positive for catalase and oxidase. Phenotypic and biochemical characteristic analyzed were showed in **Table 1**.

3.2. Detection of catabolic genes

Positive PCR amplification with alkBwf-alkBwr, TS2S- deg1RE, and/or P450F-P450R couple primers evidenced the presence of alkane-monoxygenase (*alkB*) and/or cytochrome *P450* genes, while the biphenyl dioxygenase (*bphA*) didn't show amplification in the strains in study. The presence /absence (positive/ negative amplification) of these genes is an indirect index of potential capability of the strains in study of degradation of hydrocarbons, but in the same time, it isn't express efficiency in degradation process.

3.3 Biosurfactant production and emulsification activity

277 The results obtained from the Surface Tension, drop collapse, oil spreading test and
278 Emulsification Activity (E24%)
279 have been reported in the **Table2**. The *thalassospira* spp. strains are producers of biosurfactants
280 and showed a good emulsification activity. Data obtained with drop collapse test identified
281 positive results for all isolates, but this contrast with data were obtained to other test. This
282 mismatch can be absolutely correlated to the different type and concentration of biosurfactant
283 produced and / or to the different experimental conditions (Youssef et al., 2004; Rodrigues et
284 al 2006).
285
286 *3.4. GC×GC-Q MS/FID Analyses*
287 As shown in **Figure 1**, the Arabian Light Crude Oil represents a very complex sample
288 containing different chemical classes. To achieve a reliable identification and quantification of
289 the different chemical classes the separation power of a mono-dimensional GC system is not
290 enough. For such a reason, in the present research, a GC × GC was employed due to its higher
291 peak capacity and the capability to couple columns with different selectivity. Moreover, in such
292 a kind of samples, the use of an MS detector is mandatory for reliable identification. However,
293 the drawback of this detector is the different response according to the analyte, do not allowing
294 quantification in case of lack of standard materials. For this purpose, FID is the only detector
295 providing virtually the same response according to the chemical class (Zoccali et al., 2016).
296 Thus, in the present paper, we developed a GC × GC with a dual detection system able to
297 perform both identification and quantification of the different chemical classes in a single run.
298 The goal of the GC × GC method optimization is to occupy as much as possible of the
299 two-dimensional space (without occurring in the wrap around effect) by achieving the best
300 separation possible and an ordered chromatogram. A medium polar column (35%) was used in
301 the first dimension and a non polar column (5%) was used in the second dimension, with such

a kind of configuration the non-polar components will occupy the upper part of the plot, while the more polar ones the lower region. Compounds identification were carried out according to MS spectral similarity, linear retention indices, and 2D position. In **Figure 1**, reporting the Arabian Light Crude Oil analysis, the upper part of the 2D space was occupied by linear and branched alkanes forming a fairly horizontal row of spots, and cyclic alkanes are positioned below the row of the *n*-alkanes. As can be seen in **Figure 1** the retention in the second dimension decrease according to the increase of the aromatic ring number, while increase in the first dimension also considering the length of the alkyl chain. The use of the developed GC×GC-Q MS/FID method, has allowed the quantification of the residual HC after incubation of the different bacterial *Thalassospira* isolates. Totally, 6 different chemical classes were evaluated namely: *i*) linear and branched alkanes, *ii*) cyclic alkanes, *iii*) C6-aromatics, *iv*) di-aromatics, *v)* tri-aromatics + sulphur compounds and *vi*) tetra-aromatics + sulphur compounds. All the analysis were carried out by using heptamethylnonane as internal standard and the values were calculated considering the GC×GC-FID response. The obtained results were reported in the histogram in **Figure 2**, expressed as percentage of degradation. Considering the total contamination interesting result were obtained for all the studied bacterial strains, in fact the degradation ranged of Arabian Light Crude Oil was between 77-91%. A important results was obtained in the microcosm inoculated with the strain affiliated to *T. xianhensis* that gives the best results with a % degradation ranging between 87% for the Cyclic alkanes and 94% for Tetra-aromatics + Sulphur compounds. These compounds, in particular, Tetra-aromatics, are recalcitrant, toxic, persistent and this result could be used for future study.

4. Discussion

Increasingly of environment pollution had involved scientific community to extend the research in this sector and in particular in bioremediation and biorecovery strategies. This

"new" technology exploits microorganisms' catabolism; indeed this last uses the pollutant compound as carbon source and energy. Various studies demonstrated as this microbial process converts pollutant compounds in atoxic or less toxic products, and as the bioremediation technologies are one of benefit strategies to recovery polluted environment. Low cost and high efficiency of bioremediation technology identify this strategy "*green technology*" and "eco-friendly" (Nicolopoulou et al, 2013, Crisafi et al., 2016). Several data about different kind of microorganisms with hydrocarbons degrading capability are present and described a lot of in literature (Yakimov 2004; Rooling et al., 2004; Cappello et al., 2007; Catania et al. 2015; Bargiela et al. 2015). The three stains isolated in this study were affiliated to the class of *Alphaproteobacteria* and precisely to *Thalassospira* genus, particularly: isolates AU5AG4, AU3AA1 and AU6SG1 were correlated respectively to *T. xianhensis* (KT348286), *T. lucentensis* (KT348281) and *T. profundimaris* (KT348287). The focus of this work has been the evaluation to features of this three species correlated with hydrocarbon degradation capability, in particular were carried out test of biosurfactant production, presence of specific catabolic genes involved in biodegradation processes (*alkB, CYP450* and *bphA* genes) and biodegradation test in microcosms and analysis of the hydrocarbons fractions degraded. Several test of biosurfactant production are detected, because these molecules have different properties, besides low concentrations of this molecules could give incorrect values (Mahjoubi et al 2013). Results of drop collapse and oil spreading test showed that all isolates in study are biosurfactant producers; however, divergent data are obtained, in particular data of superficial tension put in evidence that the isolate AU5AG4 *T. xiahnensis* is the best producer of biosurfactant, while Emulsification activity (E24%) showed good results for AU6SG1 *T. profundimaris*. Zhan et al. (2017) in their study isolated from *T. profundimaris* a siderophore affiliated to Thalassomide family. These results could be dependent by the nature of biosurfactant, by the concentration of this molecule and to the test applied. In the study of Youssef et al. (2004) the assessment of

surface tension is time- depending and showed a correlation coefficient stronger than drop collapse and oil spreading test, which are indicated as more reliable detector of low concentration of biosurfactant. Zhang et al. (2017), in their study, reported that this widespread genus produces a siderophore affiliated to thalassosamide family, that is efficacy against *Pseudomonas aeruginosa*. Results about the analysis of catabolic genes (alkano-monoxygenase and citocrome P450) confirmed as this three bacterial species present in their metabolism hydrocarbons degrading capability. However, the rates of biodegradation of hydrocarbons substrate in the same isolates are different, in particular isolate AU5AG4 *T. xiahnensis* resulted to have better biodegradation capability than others two isolates in study. Analysis of Oil degradation evidence as the strains in study are capable both degrade linear and branches alkanes, both fractions of molecules more complex and toxic as Cyclic alkanes and Di-, Tri- and Tetra-aromatics compounds. The molecular structure of this kind of pollutants is usually difficult to covert in molecular less toxic and recalcitrant, the strains of *Thalassospira* applied in this study had able to transform and biodegradate this pollutants in few time. Recovery of oil polluted areas can be achieved by physic-chemical or biological methods. Due to negative aspect of the physic-chemical approach (Chibueze Azubuike et al., 2016), more attention is being given to the exploitation of biological alternatives (Okoh, 2006). Biological treatments are becoming more important, mainly due of the low environmental impact, costs (in general cheaper than other cleanup technologies), the capability to destroy/degrade organic contaminants and the possibility of advantageous use of treated sediments (Rulkens and Bruning, 2005). Several studies have shown better results using bioremediation strategies (Beolchini et al., 2010; Rocchetti et al., 2011, 2012; Cappello et al., 2015). In general, bioremediation is often based on *in situ* stimulation of the microbial community (biostimulation) or amending the microbial community with an inoculum of hydrocarbon-degrading bacteria (bioaugmentation). In both cases, the successful result of bioremediation

depends on appropriate hydrocarbon-degrading consortia and environmental conditions. In this study, interesting results suggest as the isolates of *Thalassospira* species are important and relevant not only in the next studies of biotechnological processes, as the production and the extraction of significative biomolecules used in several industries including organic chemicals, petroleum, petrochemicals, mining, metallurgy (mainly bioleaching), agrochemicals, fertilizers, foods, beverages, cosmetics, pharmaceuticals and many others (Vijayakumar S. and Saravanan V. 2015), but also in the understanding of the bioremediation processes and in the application of the same microorganisms in bioremediation techniques (Scaffaro, et al., 2017, Catania et al., 2020).

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Conflict of Interest

No conflict of interest is declared by the authors and none of the authors of this paper has a direct financial relationship with the commercial identities mentioned in this article.

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611 **Tables**

612

613 **Tab. 1.** Closest relatives of the 16S rRNA gene sequences of bacteria isolated in this study and
614 results of the main classical biochemical.
615

616

Isolate code	Closest type strain	Identity (%)	Oxidase test	Catalase test	Mobility	Shape
AU5AG4	*T. lucentensis*	98	Positive	Positive	Yes	Curved-rod
AU3AA1	*T. profundimaris*	99	Positive	Positive	Yes	Curved-rods to spirilla
AU6SG1	*T. xianhensis*	98	Positive	Positive	no	Curved-rod

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Tab. 2. Superficial Tension, Drop Collapse test, Oil spreading test and Emulsification Activity of strains in study. Positive data of Drop Collapse and Oil spreading test are indicated by the symbol "+", in contrast negative data are indicated with the symbol "-".

Isolate code	Closest type strain	Superficial tension (mN m-1)	Drop collapse test	Oil spreading test	E24 test (%)
AU5AG4	*T. lucentensis*	49.7 ±0.5	+	+	22.2 ±0.5
AU3AA1	*T. profundimaris*	53.8 ±0.5	+	+	21.9 ±0.5
AU6SG1	*T. xianhensis*	53.2 ±0.5	+	+	11.1 ±0.5

Figure Captures

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence analysis of isolates in study (AU3AA1, AU5SG4 and AU6SG1) and relationship with different species of the genus Thalassospira, some genera related to the class of Alpha-proteobacteria (Terasakiella and Aquaspirillum), and others hydrocarbons degrading bacteria affiliated to the class of Gamma-proteobacteria. As Outgroup was used the 16S rRNA gene of Methanococcus jannaschii (M59126).

Fig. 2. GC×GC-Q MS analysis of Arabian Light Crude Oil. Abbreviations: B, benzene; N, naphtalene; F, fluorene; DBT, dibenzothiophene; P, phenanthrene; Py, pyrene; BNT, benzonapthothiophene; Cry, chrysene; Per, perylene. The abbreviations C1, C2, etc., refer to the number of carbons in the alkyl substituents. The intensity of the peaks is related to color (white minimum, red maximum).

Fig. 3. Rate (%) of biodegradation (calculated by using the GC×GC-FID response) of the different bacterial strains studied in the present work: isolate AU6SG1 (T. profundimaris; white bars), isolate AU3AA1 (T. lucentensis; light gray bars) and isolate AU5AG4 (T. xianhensis; dark gray bars).