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Isolation and Characterization of Oil-Degrading Bacteria from Bilge Water

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Abstract

Twenty-one oil-degrading bacteria were isolated from bilge water. Based on a high growth rate on crude oil and on hydrocarbon degradation ability, 7 strains were selected (from 21 isolated) for further studies. 16S rRNA gene sequencing showed that isolated strains were affiliated to *Bacillus, Pseudomonas* and *Halomonas* genera; in particular, isolate BW-B12 (*Bacillus sp.*, 99%), BW-C12 (*Halomonas boliviensis*, 99%) and BW-E12 (*Halomonas boliviensis*, 98%) were the best crude-oil degraders; after 10 days of cultivation in ONR 7a mineral medium supplemented with crude oil as single carbon source BW-B12, BW-C12 and BW-E12 showed a degradation rate of 80, 60 and 59%, respectively. The strains showed also a high emulsification activity and biosurfactants production. Obtained results give an important contribution in order to utilize these bilge water autochthonous microorganisms in processes of bioremediation of marine environment chronically polluted from saline oily waste.

Keywords

Bilge Water, Bioremediation, Marine Pollution, Oil Degrading Bacteria, Saline Oily Waste

1. Introduction

Bilge waters comprise about the 10% of total oils ([1], [2]) that are annually illegally discharged from the vessels into the sea ([3]).

The bilge is the lowest compartment of a ship, below waterline where rain and seawater, entering in the hull of the boat, are accumulated. In the bilge are principally converted waste products of ship operations, as well fuel, lubricants and engine oil ([4]). The mixture derivated by union of all these substances is known as "bilge water".

In particular bilge (waste) water generally includes lubricating oil, cleaning diesel oil, oily sludge, spills from engine room, water leaks from internal pipes and sea water filtrations ([1]). Bilge water may also contain various oxygen-demanding substances, volatile and semi-volatiles organic compounds, inorganic salts, metals and other contaminant materials such as soaps, detergents, dispersants, and degreasers used to clean the engine room (EPA 842-R-07-005).

Direct discharge in sea of untreated bilge water is obviously illegal and several treatment methods should be considered before. The Annex I of the International Convention for the Prevention of Pollution from Ships (MARPOL) includes accurate recommendations for wastes treatments.

Therefore, different alternative technologies have been proposed to treat bilge water in order to maintain international standard discharge levels, such as wet air oxidation ([5]), ultra/filtration ([6]), incineration. On the other hand, methods based on conventional gravity of oil separators are unable to reach the requirements of the International Maritime Organization (M)Resolution MEPC.107(49) (MEPC 49/22/Add.2, 2003), because simple gravity of oil separators often have low effect on the emulsified bilge water

produced on board ([6]). Development and optimization of bilge waste treatments to minimize costs are a research topic of worldwide interest ([1]).

The use of bioremediation technologies provides to identify a process of naturally conversion of chemicals and/or petrochemicals substances into non-toxic byproducts without any further local environmental damage ([7]).

Cleaning bilges and bilge waters is possible using "passive" methods such as bioremediation. This process is based on use of nutrients or aeration to enhance the activity of indigenous organisms (*biostimulation*) and/ or the addition of microbial inocula (*bioaugmentation*) to enhance the clean-up processes ([8]).

The possibility to identify and to isolate native bilge water microorganisms capable to degrade a high variety of hydrocarbons could be a very interesting way for possible application in cleaning processes and exploitation in future biotechnological applications ([7]). As indicated, the bilge wastewaters are extremely heterogeneous matrices; this hyper-variability in composition determines a wide variety of microbial populations that can be isolated.

In the present work different oil-degrading bacteria were isolated and characterized from bilge water and the abilities of these strains to produce biosurfactant and to biodegrade crude oil were evaluated.

2. Material and Methods

2.1. Sample Collection

A bilge water sample (5000 ml) was collected, in December 2011, from a private passenger ship, temporarily located in the harbour of Milazzo (38°13'04.35"N – 15°14'28.88"E; Tirrenean Sea, northern Sicily, Italy). The sample was collected, in sterile conditions, using glass bottles. The sample was immediately transported to the laboratory (30 min) in a cool box (4±1°C) and used for further analysis.

2.2. Physical-Chemical Characteristics

Main physical-chemical parameters (pH, temperature, salinity, redox) were measured using a multiparameter probe Waterproof CyberScan PCD 650 (Eutech Instruments, The Netherlands) and biochemical Oxygen Demand (BOD_5) were analyzed using a BOD sensor (Velp Scientifica Milan, Italy) according to the manufacturer's instructions.

2.3. Hydrocarbons Analysis of Bilge Water

The composition of total extracted hydrocarbons and their derivates (TERHCs) was obtained from 1 L of sample, by high-resolution GC–FID (*DANI Master GC* Fast Gas Chromatograph System, DANI Instruments S.p.A., Milan). Hydrocarbons were quantified according to previously described protocols ([9]).

2.4. Plate Counts

For enumeration of total heterotrophic bacteria and hydrocarbon-degrading bacteria, bilge waste samples were serially diluted in sterile physiological solution and plated on Marine agar 2216 medium (Difco S.p.a, Milan, Italy), ONR7a mineral medium ([10]) and ONR7a supplemented with sterile bilge waste (100 μl) as single carbon source, placed on the cover of the Petri plate. Culture media and bilge water were autoclaved separately for 20 min at 120°C. All agar plates were incubated at 25±1°C for 7 days.

2.5. Isolation of Microbial Strains

ONR7a mineral medium was used for the isolation of oil-degrading bacteria. Single colonies obtained from count of cultivable bacteria grown in ONR7a medium with bilge waste, were selected and purified by repeated streaking under identical growth conditions. ONR7a media were supplemented with sterile 1% (v/v) crude oil (Arabian light crude oil) as the sole carbon source.

Phenotypically different colonies obtained from the plates were purified and transferred to fresh medium with and without crude oil to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on crude oil were stored in stock media with glycerol at 20°C for further characterization ([11]).

2.6. 16S rDNA Gene Sequence Determination and Analysis of Phylogenetic Relationships

Analyses of the 16S rDNA gene sequences of isolates were performed as previously described ([12]). Total DNA extraction of bacterial isolates was performed with the CTAB method ([13]). 16S rDNA loci were amplified using 16S rDNA forward domain-specific Bacteria, Bac27_F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer Uni_1492R(5′-TACGYTACCTTGTTACGACTT-3') [14]. The amplification reaction was performed in a total volume of 50 μl mixture containing 1× solution Q (Qiagen, Hilden, Germany), 1 × Qiagen reaction buffer, 1 μM of each forward and reverse primer, 10μM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), 2.0 mL (50-100 ng) of template and 2.0 U of Qiagen Taq Polymerase (Qiagen).

The amplified 16S rDNA was sequenced using Macrogen Service (Korea). SIMILARITY_RANK from the Ribosomal Database Project (RDP) ([15]) and FASTA Nucleotide Database Query ([16]) were used to determine partial 16S rDNA sequences to estimate the degree of similarity to other 16S rDNA gene sequences. Analysis and phylogenetic affiliates of sequences was performed as previously described ([17], [18]).

2.7. Growth Conditions and Crude-Oil Removal Assay

Started cultures were carried out by inoculating one loop of microbial cells into 10 mL of ONR7a mineral medium containing 0.1% (w/v) of sterile Arabian light crude oil. After growth in a rotary shaker (Certomat IS B. Braun Biothec International, 120 ×g) at 25±1°C for two days, 500 μl of the

seed culture broth were transferred into a 500 ml Erlenmeyer flask containing 250 mL of ONR7a medium supplemented with 1% (w/v) of sterile Arabian light crude oil. The culture was incubated in a rotary shaker (120 ×g at 25±1°C) for 10 days. A flask, under the same conditions without microorganisms, served as the abiotic control. All experiments were carried out in triplicate.

The growth of the isolates was routinely assessed indirectly by measuring the turbidity (OD_{600nm}) using a UV–visible spectrophotometer (Shimadzu UV-160, Japan).

The crude oil removal assay was carried out by dissolving the residual crude-oil in the medium in dichloromethane (Sigma-Aldrich, Milan) and reading the optical density of the oil extract against a blank at a wavelength of 420 nm ([11], [18]).

2.8. Measure of Biosurfactants Production, Emulsification Activity and Liquid Surface Tension of Obtained Isolates

During growth in ONR7a medium with crude oil, biosurfactant production, emulsification activity and liquid surface tension were measured.

Biosurfactants production (drop collapse test) was performed according to the official protocol ([20]).

Emulsification activity (E_{24}) was determined by addition of hexadecane to an equal volume of culture broth; than mixture was vortexed for 2 min and then left to stand for 24 h. Emulsification activity was determined with quotient between height of the emulsified layer (mm) and total height of the liquid column (mm).

Surface tensions of different bacterial cultures were measured (after 10 days of growth), according by the Wilhelmy plate method that uses a digital tensiometer (Gibertini, Italy), in agreement with the manufacturer's instructions. It was expressed in units of mN m^{-1}.

3. Results

3.1. Physical-Chemical Parameters of Bilge Water

Table 1 shows data obtained from measure of pH, temperature, salinity, redox and Biochemical Oxygen Demand (BOD_5) for the bilge water analyzed.

3.2. Hydrocarbons Analysis

GC-FID analysis underlines as the total hydrocarbons present in of bilge water in study corresponding at ~7000 ppm. The hydrocarbons profile corresponding to the *n*-alkane homologous series (from C_{10} to $C_{40;}$ ~1100 ppm) and PAHs (~6.7 ppm). A rate of ~3000 ppm corresponding to Unresolved Complex Mixture (UCM); the residual amount of hydrocarbons is related to "mineral oil".

3.3. Count of Cultivable Bacteria

Data related to the cultivable bacteria fraction on different culture media (Marine Agar, ONR7a and ONR7a with addition of bilge water as only carbon and energy source) are shown in table 1.

Table 1. *A, Principal physical-chemical values obtained from bilge water in study. B, values of cultivable bacteria fraction on different culture media. Temp., temperature; Sal., salinity; BOD₅., Biochemical Oxygen Demand; MA, Marine Agar medium; BW, bilge water.*

	Bilge Water	
(A)	pH	6.89 ± 0.5
	Temp. (°C)	21 ± 1
	Sal. (gr L^{-1})	32 ± 1
	Redox (mV)	197 ± 5
	BOD_5 (ppm O_2 L^{-1})	36-7 ± 0.5
(B)	CFU (MA)	3×10^3
	CFU (ONR7a)	2.5×10^2
	CFU (ONR7a + BW)	2×10^3

3.4. Isolation of Microbial Strains

Twenty-one (21) bacterial strains were isolated from bilge water. Seven (7) isolated strains that showed higher growth rates on crude oil were for further studies.

The molecular characterization of isolates was carried out by amplifying and sequencing the 16S rRNA gene and comparing the sequences to the database of known 16S rRNA sequences. Taxonomic analysis of 16S rRNA (Figure 1) shows that two isolates indicated as BW-H11 and BW-D12 are related (98 and 99% of identity, respectively) to strain *Pseudomonas aeruginosa* strain NBAII AFP-4 (FM957532); isolates BW-H12 and BW-A12 are clustered with *Halomomans boliviensis* LC1 (AY245449) (99 and 98% of identity, respectively) and the strain named BW-B12 showed 99% of similarity with *Bacillus* sp. 10403023 (JF824810). Isolates BW-C12 and BW-E12 were related to *Pseudomonas stutzeri* strain JMC01 (FM957535) (99 and 98% of identity, respectively).

The sequences of the 7 studied bacteria were submitted to the genetic sequence database at the National Center for Biotechnical Information (NCBI).

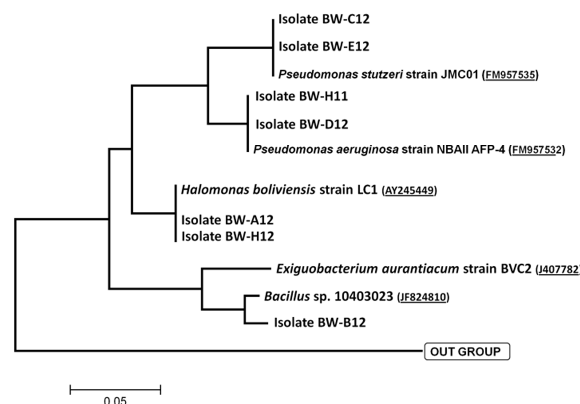

Fig. 1. *Phylogenetic tree based on 16S rRNA gene sequences for bacterial strains (isolate BW-A12, BW-B12, BW-C12, BW-D12, BW-E12, BW-H12 and BW-H11) isolated from the bilge water used in this study. Percentages of 100 bootstrap re-sampling that supported the branching orders in each analysis are shown above or near the relevant nodes (●, values >85%; o, values <85%). Evolutionary distance is indicated by vertical lines; each scale bar length corresponds to 0.05 fixed point mutations per sequence position.*

3.5. Growth Rate and Crude-Oil Removal by the Studied Strains

All bacterial strains grew in ONR 7a supplemented with crude oil (1%) for 10 days with shaking. After the incubation period, the levels of microbial growth and crude oil biodegradation were analyzed using spectrometry-based methods.

Table 2. *Growth and crude oil removal by strains.*

Isolate Code	Growth rate (OD_{600nm})	%, oil removal
BW-B12	0.70	80 ± 5
BW-H11	0.45	36 ± 5
BW-D12	0.4	37 ± 5
BW-H12	0.16	32 ± 5
BW-A12	0.2	29 ± 5
BW-C12	0.65	60 ± 5
BW-E12	0.60	59 ± 5

As reported in table 2, strains BW-B12, BW-C12 and BW-E12 exhibit highest levels of crude-oil biodegradation, degrading 80%, 60% and 59% of hydrocarbons, respectively. The strains BW-H12 and BW-A12 exhibit the lowest growth rate and crude-oil degradation levels (32% and 29%, respectively).

3.6. Measure of Biosurfactant Production, Emulsification Activity and Liquid Surface Tension of the BW Isolates

Drop collapse method was performed to analyze biosurfactant production on studied strains. In addition to emulsification activity and surface tension, reduction assays were performed for each strain. Obtained results are shown in table 3. Isolate BW-12 exhibits a lower emulsification activity than other strains (30 ± 5 %) and a superficial tension decrement of 37 ± 0.5 mN m^{-1}; on the contrary isolate BW-H11 and isolate BW-D12 exhibit the highest emulsification activity (45.1 ± 5 % and 46.3 ± 5 %, respectively) with a superficial tension decrement of 52.1 and 51.9 ± 0.5 mN m^{-1}, respectively. Intermediate values of emulsification activity and superficial tension decrement were observed in other analyzed strains. Biosurfactant production was relatively dependent to E_{24} test and superficial tension values.

Table 3. *Measurement of biosurfactant production, emulsification activity (%, E_{24}) and the decrease in the surface tension (nN/m) for each strain included in the study. Surface tension of ONR medium without bacteria as blank is 53.6 ±0.5.*

Isolate Code	Drop Collapse Test	E_{24} Test	Superficial Tension
BW-B12	+ +	30 ± 5	37 ± 0.5
BW-H11	+	45.1 ± 5	52.1 ± 0.5
BW-D12	+	46.3 ± 5	51.9 ± 0.5
BW-H12	+ +	42.2 ± 5	48.3 ± 0.5
BW-A12	+ +	42.3 ± 5	48.2 ± 0.5
BW-C12	+ +	40.7 ± 5	39.2 ± 0.5
BW-E12	+ +	40.9 ± 5	38.3 ± 0.5

4. Discussion

Bilge wastewaters are extremely heterogeneous matrices and generally include lubricating oil, cleaning diesel oil, oily sludge, spills from engine room, water leaks from internal pipes and sea water filtrations ([21]); this hyper-variability in composition could favor the presence of a wide variety of microbial populations able to be isolated and cultivated.

Possibility to operate a selection and identification of microorganisms naturally present in bilge waters is a very important point of interest for cleaning processes of polluted wastes. Different authors ([22], [23], [24]) isolated various microorganisms from bilge water samples (e.g. *Rhodococcus erythropolis*, *Acinetobacter* sp*., Pseudomonas mendocina).* They isolated some oil-degrading bacteria affiliated to genera *Pseudomonas*, *Bacillus* and *Halomonas*.

In this study, 21 bacteria were isolated from bilge water sample. Seven strains were selected for further study based on their high growth rates on crude oil and high levels of hydrocarbon degradation.

Strains related to *Bacillus sp.* (isolate BW-B12) and *Halomonas boliviensis* (isolates BW-C12 and BW-E12) expressed also ability to produce surface active molecules. Data obtained from surface tension measures, are according to data generally present in literature. Indeed many microorganisms, such as *Halomonas sp*., *Bacillus sp*. and *Pseudomonas sp*., produce biosurfactants in environments dominated by the presence of oil hydrocarbons ([25], [26], [27]). Since these substrates are not miscible with water, their uptake requires morphological and physiological modifications, notably in cell adhesion properties (surface hydrophobicity) or in the production of emulsifiers ([28]); biosurfactants can reduce both surface and inter-surface tension, providing a great aid in emulsification processes ([11]) and enhancing the degradation of crude oil and other hydrocarbons ([29]).

5. Conclusion

Results obtained in this paper show isolation and characterization of different oil-degrading bacteria from bilge water. Use of these bacteria for bioremediation purposes will facilitate the better management of chronically polluted matrix; in particular, these isolation may improve the direct application of these strains in aforesaid biotechnological systems and optimize the performance compared to the use of non-autochthonous strains for the recovery of this type of pollution (bilge water).

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