Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D4.7
Comprehensive database of all strains, additives, formulations

This project is supported by the European Union under the Food, Agriculture and Fisheries and Biotechnology theme of the 7th Framework Programme for Research and Technological Development under GA no. 312139

Work package	WP4
Deliverable no	D4.7
Deliverable title	Comprehensive database of all strains, additives, formulations
Due date:	2016-11-30 (Month 48)
Actual submission date:	2016-12-17 (Month 48)
Start date of project:	2013-01-01
Deliverable Lead Beneficiary (Organisation name)	CNR
Participant(s) (Partner short names)	TUC, UNIBO, CSIC, CNR, Bangor, UMIL, Bangor, UCL, MADEP, ACTY
Author(s) in alphabetic order:	Agathos, Aulenta, Beffa, Beltrametti, Borin, Cappello, Fava, Ferrer, Kalogerakis, Mapelli, Raddadi, Siegl, Scoma, Yakimov, Zanaroli,
Contact for queries:	Michail Yakimov IAMC-CNR Spianata San Raineri, 86 98122 Messina, Italy T +39 090 6015437 E michail.yakimov@iamc.cnr.it
Dissemination Level: (PUblic, Restricted to other Programmes Participants, REstricted to a group specified by the consortium, COnfidential only for members of the consortium)	PU
Deliverable Status:	final

Table of Content

List of Figures

List of Tables

1 Introduction

In relation to oil spill remediation there is a growing body of evidence that microorganisms are capable of doing the job of the natural clean-up in marine environments. Within the past two decades, the new physiological group of marine oil-degrading bacteria, which rely on hydrocarbons as their only source of carbon and energy and inability to utilise common substrates (sugars and amino acids) has been characterised. These so-called obligate hydrocarbonoclastic bacteria (OHCB) (Yakimov et al., 2007) have been isolated in pure cultures and characterised as new genera and families within (mostly) gamma-proteobacterial class. Surprisingly, they have later been detected in (or isolated from) polluted or oil-impacted marine environments across the world ocean, at various depths, temperatures and redox conditions. Historically, the degradation studies focussed on non-marine rhodococci, pseudomonads, burkholderia of acinetobacter species, therefore in the context of potential applications in marine environments it is important that OHCB, the genuinely marine microorganisms, are readily adaptable to the marine environments.

Despite recent success in isolation of new oil-degrading microorganisms with a potential for applications in bioremediation, there is a strong demand in expanding the array of strain and cultures capable to tackle the pollution. The huge chemical diversity and complexity of compounds constituting petroleum and the finite sizes of individual microbial genomes with limited numbers of degradation pathways do dictate the necessity in isolation of new microorganisms and their consortia. Especially neglected in this context are marine fungi, studies on which contribute only a minor fraction to the body of literature on oil-degrading microorganisms despite their great potential in biotechnological applications and their extensive use in the past in the “food-from-oil” technologies of 1960-70s.

2 Relevant tasks

In the Workpackage 4 of the Kill-Spill project the deliverable 4.7 was specifically addressed in the **Task 4.1:** *Isolation of MOs from environmental matrices,* which had two Objectives:

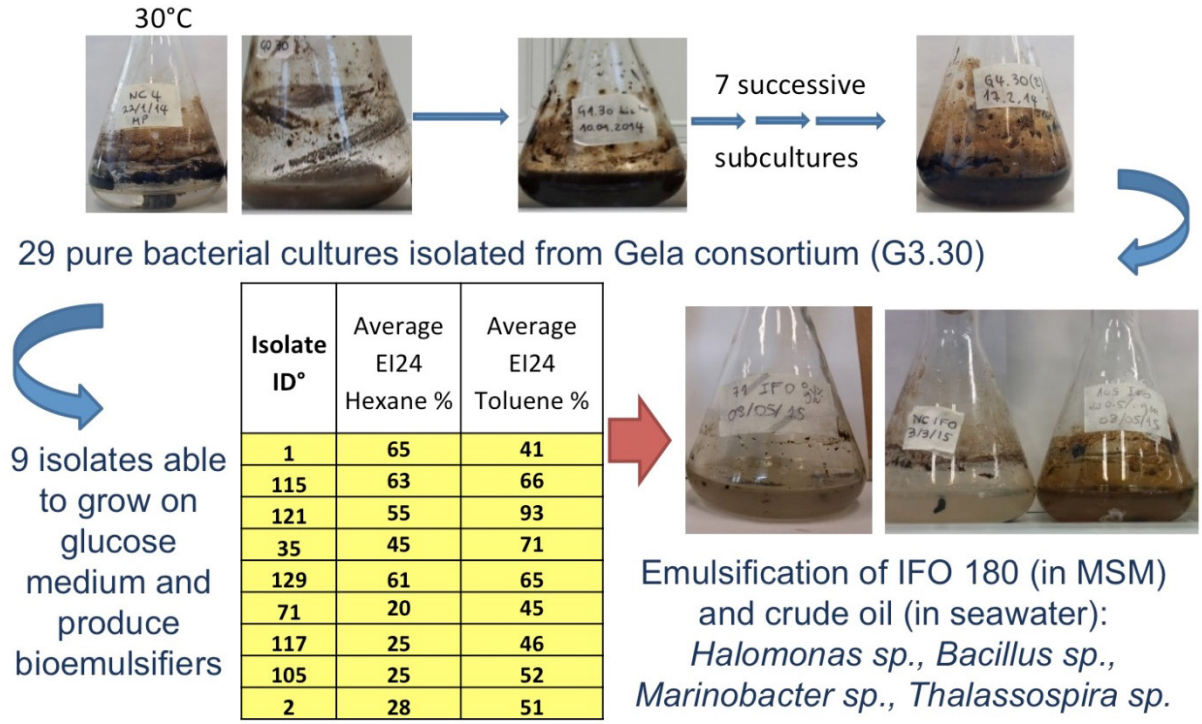
- Isolation and selection of aerobic consortia with bioremediation potential
- Isolation and characterization of hydrocarbon degrading fungi and evaluation of fungal or bacterial consortia

with an overall aim to obtain both single hydrocarbon degrading isolates and hydrocarbon-degrading consortia (enrichments) from oil-impacted environments and field sites.

3 General methodologies

To obtain cultures of oil-degraders and their consortia the Project Partners used few different approaches to address this, for example some focussed on the isolation of marine aerobic organisms and their consortia (**Subtask 4.1.1: Isolation and selection of aerobic consortia with bioremediation potential),** using artificial seawater medium (ONR7a) for isolation. Samples originated from a great range of environments including *inter alia*: the Tyro deep hypersaline anoxic basin (DHAB), Ancona, Menai Straights, Augusta bay, Gela shore after the oil-spill that occurred on 4th June 2013 in the proximity to the ENI refinery and many others. The microcosms were initially established using ONR7a medium supplemented with crude oil. These were incubated at 30°C in the dark, until turbidity was observed. Afterwards each microcosm was split and re-inoculated in three separate vials, these were incubated with different hydrocarbons (octane, dodecane) or crude oil as sole

carbon source. Other partners were chasing up the soil oil-degrading bacteria, with the emphasis on emulsifier-producers, and thirdly, on the fungal component of oil-degrading community.



Isolate ID°	Average EI24 Hexane %	Average EI24 Toluene %
1	65	41
115	63	66
121	55	93
35	45	71
129	61	65
71	20	45
117	25	46
105	25	52
2	28	51

Figure 1 **A general scheme of the workflow for isolation of microorganisms** from enrichment cultures exemplified on the use of polluted sediments (Gela site) as a source, which after consecutive re-inoculations of media with IFO180 crude oil and plating has yielded 29 isolates, some of which were capable of bioemulsifiers' production**.**

3.1 Sources of isolates/enrichment cultures

Fifteen different environments have been exploited for strain isolation, covering a wide range of conditions: marine (seawater, sediments), terrestrial (soil, rhizosphere, sand) and industrial (harbours, oil producing wells) environments, and both pristine and hydrocarbon contaminated. Extreme environments characterized by dryness and hypersalinity (Chotts, desert, Mediterranean deep hypersaline anoxic brines) have been, moreover, specifically targeted for the isolation of strains able to degrade hydrocarbons and/or produce biosurfactants.

3.2 Microbial diversity

The primary collection across all Kill•Spill partners was more than 100 strains obtained in pure cultures. New isolates have been identified by molecular methods: sequencing 16S rRNA gene (Bacteria) and ITS (Fungi). The strains have been identified at species/genus level and most of them belonged to the previously described taxa. Eighty-four strains belong to the domain bacteria, while 14 isolates belonged to Ascomycetes and Basidiomycetes (fungal domain). The bacterial collection encompasses a wide taxonomic diversity, including several genera of γ-proteobacteria (52% of identified strains), Firmicutes (13%), Actinobacteria (23%), α-proteobacteria (12%). Several strains belonged to known hydrocarbonoclastic species while others were not previously described as hydrocarbon-degraders. Four strains of known hydrocarbonoclastic bacteria earlier isolated by partners BANGOR and IAMC-CNR were for benchmarking purposes.

3.3 Performance of isolates and consortia

Microbial pure and mixed cultures were typically characterised for their performance with both, selected constituents of oil and with the crude oil of the light and heavy types. The performance was monitored in laboratory micro- or mesocosms, by assessing biomass production (cell numbers/protein in the biomass/total biomass carbon), and measuring residual concentrations of substrates (gravimetry and/or gas chromatography-mass spectrometry). In some cases, the bioemulsifying activity was also monitored in the course of degradation experiments as shown in Figure 3. Similar analyses have been obtained for most of other strains and consortia.

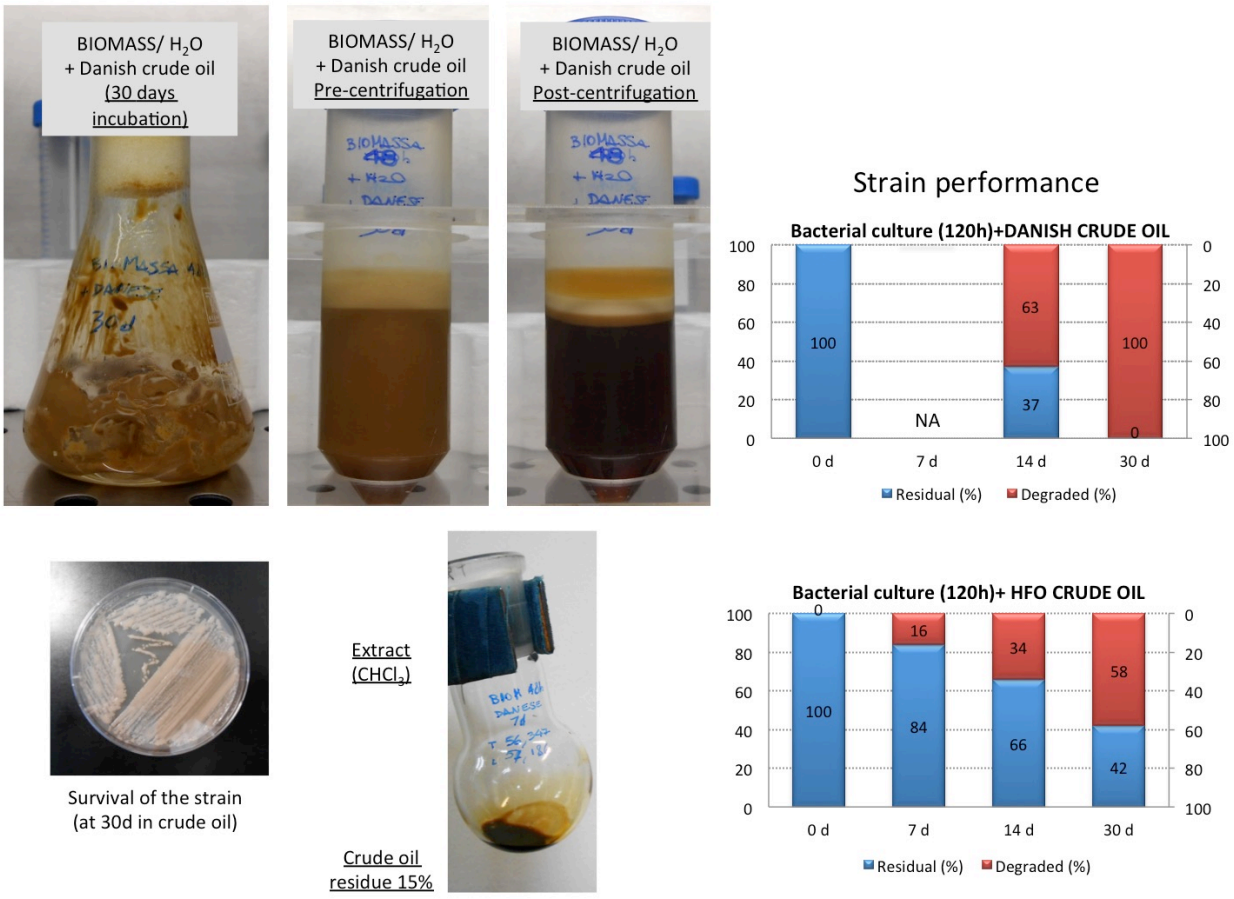


Figure 2 **Strain performance exemplified by *Rhodococcus* HFO2B.**
Typically, the measurements of growth (biomass, cell numbers) parameters were accompanied by the viability tests, emulsifiers' production and analysis of residual oil concentrations in the culture (gravimetry of chloroform extracts)

4 Concluding remarks

- Kill•Spill Consortium has produced the collection of pure cultures and microbial consortia of marine and terrestrial microorganisms capable to degrade hydrocarbons (light and heavy crude oil and individual hydrocarbon fractions)
- The sources of isolation were more than fifteen environments either oil-polluted or laboratory micro/mesocosms spiked with hydrocarbons
- Majority of bacterial isolates belonged to the established genera, many of which, however, have not previously been attributed to the oil-degraders
- A large fraction of microbial formulations has been tested for their performance with eight identified being the most promising
- Strains and mixed cultures can be made available upon request (subject to negotiation with indicated Beneficiaries)

The data on strains and consortia is summarised in the **Table 1** with the description of possible application(s. below).

5 References

1. Yakimov MM, Timmis KN, Golyshin PN. (2007) Obligate oil-degrading marine bacteria. Curr Opin Biotechnol. 18:257-266.

Table 1 The census of 119 microbial strains and consortia obtained in the course of the Kill-Spill Project featuring tested applications and performances. Availability of strains and consortia can be negotiated with the indicated contact persons.

Partner: UMIL, Contact: Prof Sara Borin (sara.borin@unimi.it)				
Bacterial Species	Number of isolates	Formulation	Production cost	Tested application
Alcanivorax borkumensis	20	n.a.	Not estimated	All the strains are able to grow in mineral media using hydrocarbons as the sole carbon source. The strains were also tested for their capability to: i) degrade uric acid, when supplied as a fertilizer; ii) adhere on plastic surfaces and hydrocarbons molecules, useful for application with oil sorbent materials; iii) produce bioemulsifying molecules.
Alcanivorax dieselolei	5	n.a.	Not estimated	
Alcanivorax venustensis	14	n.a.	Not estimated	
Marinobacter adhaerens	32	n.a.	Not estimated	
Marinobacter alkaliphilus	11	n.a.	Not estimated	
Marinobacter flavimaris	13	n.a.	Not estimated	
Marinobacter hydrocarbonoclasticus	5	n.a.	Not estimated	
Marinobacter hydrocarbonoclasticus strain ANU5	1	The isolate was cultivated with CYSP and Medium Acty 333 media, lyophilized and enclosed in alginate beads with the addition of nutrients and surfactants	Not estimated	The capability of the revitalized strains to degrade crude oil was tested and confirmed. Test on crude oil degradation in microcosms conditions.

UNIBO, Contact: Dr Giulio Zanaroli <giulio.zanaroli@unibo.it>;Dr Noura Raddadi <noura.raddadi@unibo.it>				
Bacterial Species	Number of isolates	Formulation	Production cost	Tested application
Halomonas sp.	1	not available	Not estimated	All marine strains are able to grow in mineral salt medium or modified (with the addition of 30 g/l of NaCl) mineral salt medium supplementeed with 2% soybean oil as major carbon source and produce biosurfactant and or bioemulsifiers. The surface active molecules from most of them are active and stable in the presence of low water activity (in the presence of up to 300 g/l of NaCl), after autoclaving and at 4°C.
Thalassospira sp.	1	not available	Not estimated	
Marinobacter sp.	7	not available	Not estimated	
Halomonas sp. 1	1	not available	Not estimated	The isolates were obtained from marine consortia enriched in the presence of crude oil IFO 180 as major carbon source. All isolates are able to grow i) on mineral salt medium supplemented with glucose as major carbon source and produce bioemulsifiers and ii) on seawater and emulsify crude oil.
Bacillus sp. 71	1	not available	Not estimated	
Thalassospira sp. 117	1	not available	Not estimated	
Marinobacter sp.	2	not available	Not estimated	
Marinobacter sp. M27.30	1	Autoclaved marine bacterial broth culture exhibiting a bioemulsification and biosurfactant activity.	Not estimated	The whole bacterial culture broth sterilized (at 121°C for 20 minutes) and stored at a 4°C to be used as biosurfactants, bioemulsifiers is stable for at least 4 months at 4°C. The autoclaved broth culture was capable of emulsifying crude oil.

Partner: IAMC-CNR, Contact: Dr. Michail M. Yakimov <michail.yakimov@iamc.cnr.it>				
Bacterial Consortia	Number of isolates	Formulation	Production cost	Tested application
Consotium PSO [Alcanivorax (73%),Winogradskiella (10%), Marinobacter (7%), Pseudoalteromonas (3%), Lutimaribacter (3%) and Cyclobacterium (3%)]	1	ONR7a medium with the addition of crude oil (0.1% w/w)	Not estimated	Consortia are able to grow in mineral media using hydrocarbons as the sole carbon source. The consortia were also tested (after encapsulation in chitosan beads) for their capability to degrdade hydrocarbons
Consortium PSM [Cycloclasticus pugetii (72%), Porticoccus hydrocarbonoclacus (25%)]	1	ONR7a medium with the addition of PHA mix (0.1% w/w)	Not estimated	Consortia are able to grow in mineral media using hydrocarbons as the sole carbon source. The consortia were also tested (after encapsulation in chitosan beads) for their capability to degrdade hydrocarbons

Partners: ACTYGEA and MADEP, Contact: Fabrizio Beltrametti (fbeltrametti@actygea.com) Trello Beffa (trello.beffa@madep-sa.com)					
Fungal strain	Formulation Spores for long time storage (up to six months)	Instructions for field application	Production costs	Tested Application	
Cladosporium pseudocladosporiodes	Bioaugmentation with medium Acty 200, production of spores amended with nutrients. Industrial pilot test performed. Ready for delivery	Suspend spores in medium Acty 200 medium in order to have 104 cfu/cm2 of surface to be treated (approximately 0.1-0.2 ml for cm2)	<10 EUR/kg	Biodegradation (>80% light crude oil in liquid medium and sand microcosms added with 50-80 g/L of light crude oil)	
Pennicilium brevicompactum	Bioaugmentation with medium Acty 334, production of spores amended with nutrients. Industrial pilot test performed. Ready for delivery	Suspend spores in medium Acty 200 medium in order to have 104 cfu/cm2 of surface to be treated (approximately 0.1-0.2 ml for cm2)	<10 EUR/kg	Biodegradation((up to 60% degradation in liquid medium added with 50-80 g/L of light crude oil)	
Amorphotheca resinae 1828	Bioaugmentation with medium Acty 345, production of spores amended with nutrients. Industrial pilot test performed. Ready for delivery	Suspend spores in medium Acty 200 medium in order to have 104 cfu/cm2 of surface to be treated (approximately 0.1-0.2 ml for cm2)	<10 EUR/kg	Biodegradation (up to 90% light crude oil in liquid medium added with 80 g/L of light crude oil)	
Bacterial Species	Number of isolates	Formulation	Production cost	Tested application	
Rhodococcus HFO2B	1	The isolate to be cultured in ACTY 333 and used as an inoculum at 10^8 CFU per mL	Not estimated	Biodegradation of >90 % Danish crude oil (50 g/L) and about 60 % of heavy fuel oil in liquid medium ACTY 333 at 28° C within 30 days	