

## STUDY ON DEGRADATION OF CRUDE OIL AND PURE HYDROCARBONS BY HALOTOLERANT BACTERIAL CONSORTIUM FROM IRANIAN COASTS

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### ABSTRACT

Bacterial consortium and five halotolerant bacteria were isolated from a hyper saline coastal area of the Persian Gulf on sea salt water medium (SSW) with crude oil as a main source of carbon. These strains grow by optimal concentration of 3 M NaCl. Optimum growth temperatures lied at 37 °Celsius. The strains could grow on a wide scope of aliphatic and aromatic (both mononuclear and polynuclear) hydrocarbons, as sole sources of carbon and energy. Quantitative measurements revealed that some strains could degrade crude oil up to 43%, and naphthalene up to 41% in culture after 3 weeks of incubation. The rates of biodegradation by all strains were enhanced with increasing NaCl concentration in the medium. It was concluded that the analyzed halotolerant bacterial consortium could contribute to self-cleaning and bioremediation of oil-polluted hyper saline environments.

**Keywords:** Oil-polluted environments, Consortium, halotolerant, biodegradation, pure hydrocarbons

### 1. INTRODUCTION

Some of the biomes on earth include hyper saline environments like natural saline lakes, salt flats, saline industrial effluents, oil fields, and salt marshes are contaminated with high levels of

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petroleum hydrocarbons. These systems have considerable economic, ecological and social value. Around the contaminated hyper saline environments, oilfields pose a special problem due to their sheer numbers all over the world and due their high salinity caused by salty brackish water (human produced water) generated during oil and natural gas extraction.

The inappropriate management by oil industries can lead serious environmental problems. Presently, more than 90% of all produced waters are re-injected, however prior to 1965–1970 most of the human produced water waste was released to the surface. Even now many small to moderate sized operators continue to release substantial quantities of human produced waters to the surface and shallow subsurface because of leaky tanks and flow lines and due to accidents. Sabkhas or coastal salt marshes are ubiquitous features in arid and semi-arid regions of the world (Australia, Central Asia and Persian Gulf). These habitats are characterized by high salinity and extensive crude oil contamination [1, 2].

In all marine ecosystems, microorganisms play an essential role in energy transfers and nutrient cycling reactions which in turn influence the existence of higher organisms in the Iranian coast marine food web. The functionality of microbial community is dependent on the capability to promptly react to environmental changes also to the anthropogenic pollution, which significantly contributes to the global changes [3, 4, and 5]. The use of marine microbial resources to promote the pollutant degradation in situ (bioremediation) has gained considerable development in the recent years [6]. Hydrocarbon-degrading bacteria have been described also in different marine habitats including some Arabian coast and they showed both psychrophilic and psychro tolerant character [7]. The identified autochthonous microorganisms are capable to degrade aliphatic and aromatic hydrocarbons even at low temperatures in the marine environment, thus they play a crucial role in the in situ biodegradation of hydrocarbons [8, 9, 10].

Halophiles are classified into three groups on the basis their optimal salt concentration for growth: slightly halophilic (1–3% w/v), moderately halophilic (3–15% w/v), and extremely halophilic (15–32% w/v) [11]. Application of microbial technologies for treating contaminated fluctuating salinity environment is limited due to the detrimental effects of salt on microbial life including disruption cell membrane, denaturation of enzymes, and low solubility of oxygen, desiccation and low solubility of hydrocarbons [12]. For this reason, it is particularly important not only to identify but also to isolate, to select and cultivate key microbial members to better study related pathways adapted to the marine life, for ecological and biotechnological

perspectives. Based on the above, the objective of this study is to develop a halotolerant bacterial consortium, which would hopefully be able to minimize the long-term damages to the environment such as those brought about by spreading, adsorption into environment and prolonged crude oil contamination.

## 2. MATERIALS AND METHODS

Object of these studies was consortia of hydrocarbon degrading bacteria (Iranian consortium, named IC) previously isolated from the Persian Gulf. No aromatic strains were analyzed before but successfully identified five aliphatic degrading strains: *Alcanivorax dieselolei*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. Because of that we decided to isolate and identify aromatic degrading bacteria.

Bacterial Isolation and identification of aromatic degrading strains

A mixed bacterial population from IC was enriched in a medium containing naphthalene as the sole source of carbon and energy. The minimal basal salts (SWS) medium used for enrichment and further experiments contained per liter [13]: 1.0 g of  $(\text{NH}_4)_2\text{SO}_4$ , 5.0 g  $\text{KH}_2\text{PO}_4$ , 0.1 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 mg of  $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$  and 1.0 mL of trace elements solution. The trace element solution contained per liter: 23 mg  $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ , 30 mg  $\text{MnCl}_4 \cdot \text{H}_2\text{O}$ , 31 mg  $\text{H}_3\text{BO}_3$ , 36 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 10 mg  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 20 mg  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 50 mg  $\text{ZnCl}_2$ , and 30 mg  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ .

Cultivable microorganisms from consortium were counted by the dilution plating method using, as a medium, the constituent mineral compounds only of the medium described by Mevarech [14] and Naphthalene (Sigma) vapor as a sole source of carbon and energy. Naphthalene is a polycyclic aromatic hydrocarbon, because of that he has not heteroatom or carries substituent. A naphthalene molecule can be viewed as the fusion of a pair of benzene rings [15]. It can be found naturally in crude oil composition. A poll of series of dilutions (until  $10^{-4}$ ) was prepared. Aliquots, 0.25 ml of each dilution was spread on the solid mineral medium in Petri-dishes and naphthalene vapor was made available as a sole carbon and energy source from 3 mg impregnated filter papers fixed in the dish lids. Dishes were sealed with parafilm tape and incubated at 37 C for 3 weeks. Five parallel plates were prepared for every dilution. The colony forming units (CFU) were counted. Strains in the pooled replicate plates were categorized

according to their colony and cell morphologies, counted and three representative colonies were isolated.

The isolates were identified by Biolog microorganism automatic systematic identification [16]. When the microorganism utilized carbon source to respiration, tetrazole oxidation-reduction staining material would be from colorless to purple. The characteristic reaction pattern or “fingerprint” of the microorganism was formed in the microbial identification plate. The cultures of the isolates in single-colony form were suspended in the IF; the transmittance (97 %) was adjusted equivalent to that of a standard (provided by Biolog™), followed by inoculation in 100- $\mu$ L quantities in each well. Biolog microbial identification system was a set of microbial identification system based on the principle of metabolic fingerprint. The plates were incubated at 30 °C and read at every 12-h intervals till 48 h using BiologMicrostation™.

#### Relationship between growth and sodium chloride concentration

Growth of bacterial isolates in SSW with crude oil and naphthalene (for separate) as sole source of carbon and energy in the presence of NaCl (1, 2, 3 and 4 Molar) was measured. Aliquots, 10 ml of the media were dispensed in test tubes, sterilized and inoculated each with 0.1 ml of a common inoculums prepared by suspending a loop of the biomass in 5 ml sterile pond-water. To exclude the possibility that traces of organic matter in the pond-water might have had served as alternative carbon sources, it was made sure in a parallel experiment that no bacterial growth occurred when crude oil or naphthalene was not provided to the culture. Tubes containing the mineral medium were provided with 3 grams of crude oil and naphthalene from impregnated cotton plugs and tightly coating the plugs with multiple layers of parafilm to prevent volatilization in the open atmosphere. In this experiment, cells may use of the amount of oxygen that was available in the sealed tubes. Plugs of tubes containing the complete medium were coated similarly. Incubation was done at 37°C (which although not optimal, yet supported satisfactory growth) for 3 weeks and growth was measured in terms of optical density at 600 nm.

#### Hydrocarbon-utilization

Different hydrocarbon sources were tested; we tried a pool of culture media containing crude oil, benzene, toluene, petroleum ether and naphthalene as sole sources of carbon and energy. A common cell suspension (Aromatic degrading consortium, ADC) was prepared from every strain by suspending a biomass from a 3-day culture in 5 ml sterile pond-water. A loopful of the suspension was streaked onto the above mineral salt medium containing 0.5% w/v of the

individual hydrocarbons. After incubation for 3 weeks at 37°C, cultures were examined for growth.

At the end of the incubation period, residual hydrocarbons were recovered from each medium aliquot by three 30-ml aliquots of diethyl ether. The combined extract was completed to 90 ml and 1 µl was analyzed by gas-liquid chromatography (GLC), using a Chrompack (Chrompack, Middelburg, the Netherlands) CP-9000 instrument equipped with a FID, a WCOT-fused silica CP-SIL-5CB capillary column, 15 m × 0.25 mm, and a temperature program which raised the temperature from 45 to 310°C at 10°C min<sup>-1</sup>. The peak areas of residual hydrocarbons were compared to the areas of the control peaks enabling the calculation of decrease percentages. The values obtained were taken as quantitative measure of the hydrocarbon biodegradation.

Each reading was the mean of three replicates, the standard deviation values were <5% of the mean values.

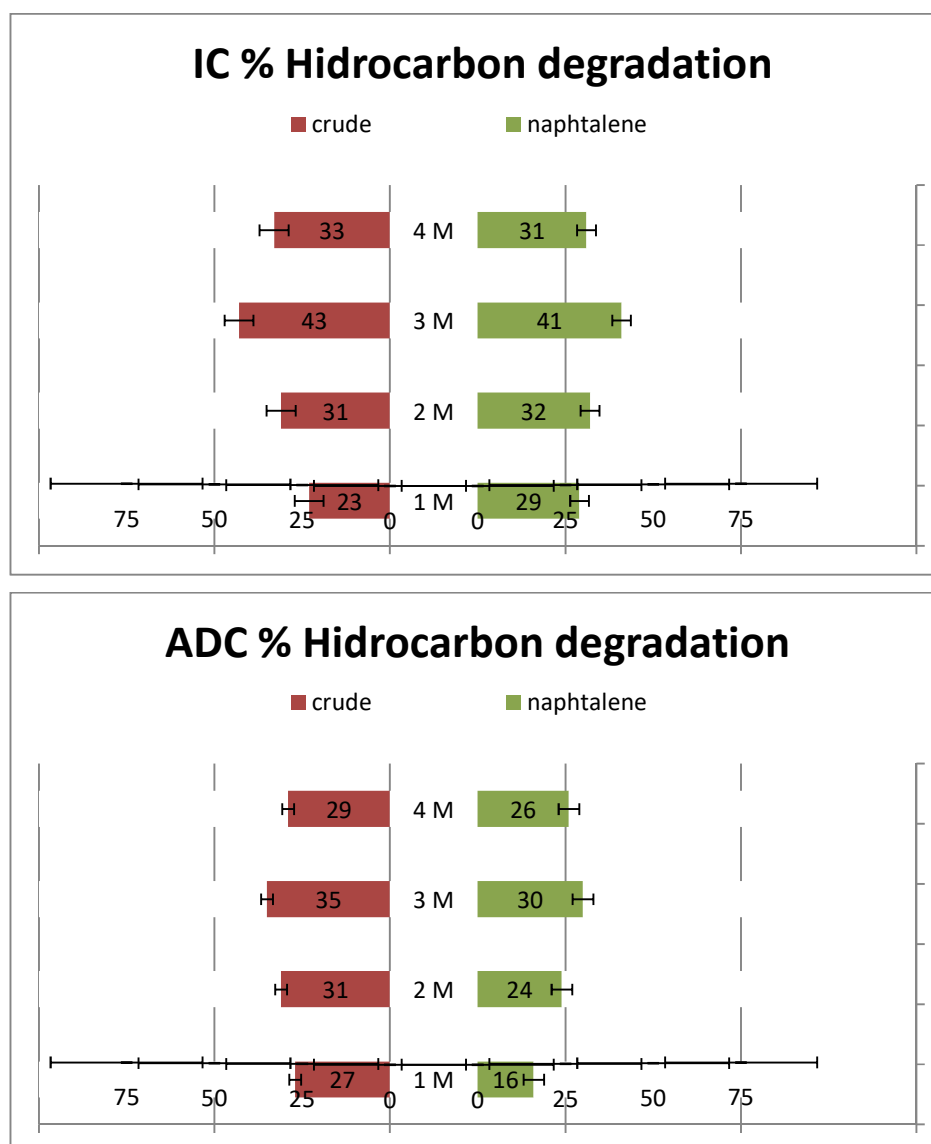
### 3. RESULTS AND DISCUSSION

Based on their capabilities to grow on naphthalene as their sole carbon source, 5 bacterial strains were isolated *Gordonia shandongensis*, *Klebsiella oxitoca*, *Ochrobacterium sp.*, *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*. These aromatic degrading strains were used in the construction of the new Aromatic degrading consortium (ADC).

Degradation of crude oil and aromatic hydrocarbons.

We compare two consortiums, Iranian consortium and aromatic degrading consortium (Figure 1), to confirm that the growth of the studied microorganisms was enhanced by increasing the NaCl concentration in the medium. Both consortiums grew best at NaCl concentration of 3 Molar.

These optima were similar irrespective of whether the carbon source was naphthalene or crude oil. The weaker level of hydrocarbon degradation might have been due to that the isolates required specific growth factors (e.g., amino acids and vitamins) which we avoided to add, or else they might have interfered with the hydrocarbons as sole carbon and energy sources.



**Fig.1.** Effect of salinity on crude oil and naphthalene hydrocarbon biodegradation by Iranian consortium IC and aromatic degrading consortium ADC. Incubation period of 3 weeks at 37°C.

#### Degradation of pure hydrocarbons

Based on their capabilities to grow on individual hydrocarbons as their sole carbon source (Table 1) we conclude that *Gordonia shandongensis*, *Klebsiella oxitoca*, *Ochrobacterium sp.* and *Pseudomonas stutzeri* appeared to be restricted to use monoaromatic hydrocarbon compounds. By the other hand we can say that *Gordonia shandongensis* and *Pseudomonas aeruginosa* are able to grow on all hydrocarbon groups tested in this study, that phenomena is described by that

microorganisms and also close to with surfactant production and degradation of xenobiotics [17, 18 and 19].

**Table 1.** Isolates used in the construction of bacterial consortia, identification and the substrates that support their growth. For isolate could use substrate as carbon source we used (+) and for that one who couldn't we used (-).

| Isolate | Isolate<br>Identification                    | Substrate |         |         |             |
|---------|--|-----------|---------|---------|-------------|
|         |  | crude oil | Benzene | Toluene | Naphthalene |
| 1.1     | <i>Klebsiella oxitoca</i>                    | –         | –       | +       | +           |
| 1.2     | <i>Pseudomonas stutzeri</i>                  | +         | +       | –       | +           |
| 1.3     | <i>Ochrobacterium sp.</i><br><i>Gordonia</i> | –         | –       | –       | +           |
| 1.4     | <i>shandongensis</i><br><i>Pseudomonas</i>   | +         | +       | +       | +           |
| 1.5     | <i>aeruginosa</i>                            | +         | +       | +       | +           |

#### 4. CONCLUSION

In conclusion, the IC and ADC halotolerant bacterial consortiums can use as sole carbon and degrade both aliphatic and aromatic hydrocarbons in the presence of 3 M NaCl. The individual strains could utilize a wide range of individual hydrocarbon sources with different chain lengths and mononuclear and polynuclear hydrocarbons. These bacterial consortiums grew optimally at elevated temperatures, 37°C. Such properties make these halotolerant microorganism suitable biological materials for bioremediation of oil-polluted hyper saline environments.

#### RECOMMENDATIONS

Is necessary a further study in crude oil reservoir to test our bacterial consortiums against field conditions like reservoir maturation, crude oil gravity over the depth and seasonal temperature range.

#### COMPETING INTERESTS

The authors declare that they have no competing interests.

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