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RESEARCH ARTICLE

Biodegradation of diesel oil by a novel microbial consortium: comparison between co-inoculation with biosurfactant-producing strain and exogenously added biosurfactants

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Abstract Bioremediation, involving the use of microorganisms to detoxify or remove pollutants, is the most interesting strategy for hydrocarbon remediation. In this aim, four hydrocarbon-degrading bacteria were isolated from oil-contaminated soil in Tunisia. They were identified by the 16S rDNA sequence analysis, as *Lysinibacillus bronitolerans* RI18 (KF964487), *Bacillus thuringiensis* RI16 (KM111604), *Bacillus weihenstephanensis* RI12 (KM094930), and *Acinetobacter radioresistens* RI7 (KJ829530). Moreover, a lipopeptide biosurfactant produced by *Bacillus subtilis* SPB1, confirmed to increase diesel solubility, was tested to increase diesel biodegradation along with co-inoculation with two biosurfactant-producing strains. Culture studies revealed the enhancement of diesel biodegradation by the selected consortium with the addition of SPB1 lipopeptide and in the cases of co-inoculation by biosurfactant-producing strain. In fact, an improvement of about 38.42 and 49.65 % of diesel degradation was registered in the presence of 0.1 % lipopeptide biosurfactant and when culturing *B. subtilis* SPB1 strain with the isolated consortium, respectively. Furthermore, the best improvement, evaluated to about 55.4 %, was recorded when using the consortium cultured with *B. subtilis* SPB1 and *A. radioresistens* RI7 strains. Gas chromatography analyses were correlated with the gravimetric evaluation of the residual hydrocarbons. Results suggested the potential applicability of the selected consortium along with the ex situ- and in situ-added biosurfactant for the effective bioremediation of diesel-contaminated water and soil.

Keywords Biodegradation · Diesel oil · Biosurfactant · Bacterial consortia · *Bacillus* sp. · *Lysinibacillus* sp. · *Acinetobacter* sp.

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Introduction

The use of enormous amounts of petroleum products contributes highly to soil and ground water contamination. Nowadays, environmental pollution by hydrocarbons receives great concern with respect to environmental and health issues. Bioremediation that involves the use of living microorganisms, bacteria, or fungi, for detoxification of soil and water organic pollutants by biodegradation, biotransformation, and/or mineralization, preferentially in situ, has been proposed as an effective technology for the control of hydrocarbons (Joutey et al. 2013). It has been recognized as powerful alternatives to conventional methods in resolving environmental problems including physical and chemical treatments (Joutey et al. 2013). Gram-negative isolates belonging to the *Acinetobacter*, *Pseudomonas*, *Alkanivorax*, and related genera are well known as the main microorganisms that can consume petroleum hydrocarbons (Mnif et al. 2014). In addition, Gram-positive isolates belonging to the *Rhodococci* and *Bacillus* genera are widely recognized by their ability to degrade hydrocarbons (Mnif et al. 2014). In fact, *Bacillus* sp. has attracted interest in both biotechnological applications and bioremediation strategies (Mnif et al. 2014).

In spite of the greatest variety of hydrocarbon-degrading bacteria, growth of certain microorganisms on hydrocarbon substrates can be limited by numerous factors like the substrate recalcitrance and the low solubility of organic compounds in aqueous systems which limited their bioavailability for biodegradation (Calvo et al. 2009; Joutey et al. 2013). Addition of surface active compounds can promote hydrocarbon solubility making them more available and enhancing therefore their biodegradation (Franzetti et al. 2009; Pacwa-Plociniczak et al. 2011). In this context, many studies have demonstrated the effect of synthetic surfactants in increasing the apparent solubility of certain hydrocarbons (Wong et al. 2004; Swe et al. 2006) and washing contaminated soil (Urum et al. 2006; Akpoveta et al. 2012). Hence, they are widely applied to improve microbial hydrophobic compounds degradation (Van Hamme et al. 2001; Noordman et al. 2002; Helmy et al. 2009; Hadibarata and Tachibana 2010; Chamkha et al. 2011). Nevertheless, the addition of persistent synthetic emulsifiers could be noxious to the environment and living organisms (Dayeh et al. 2004; Pavlic et al. 2005). Furthermore, they can inhibit biodegradation via toxic interactions and sequestration of hydrocarbons into surfactant micelles (Makkar and Rockne 2003). By against, microbial surface active compounds, characterized by their environmental friendly, reduced toxicity and biodegradability, could be considered as best alternatives to chemical surfactants in enhancing hydrocarbons solubility, bioavailability, and biodegradation.

Microbially derived surface active compounds or biosurfactants having an amphiphilic character are classified into diverse groups including glycolipids, lipopeptides, phospholipids, and free fatty acids (Fracchia et al. 2012; Shoeb et al. 2013). They reduce the surface and interface tension at water-oil interface as having both polar and non-polar domains (Fracchia et al. 2012). Owing to their surface activity and biological activities, low toxicity, high biodegradability, and environmentally acceptability, they can be applied in many domains. They are applied in food industry as emulsifying and stabilizing agents, in biomedical sciences and pharmaceutical industries as therapeutic agents to combat many diseases and in agriculture field as biocontrol agents (Fracchia et al. 2012; Shoeb et al. 2013). In addition, they are good candidates for enhanced oil recovery and bioremediation of oil spills (Pacwa-Plociniczak et al. 2011).

Besides the use of surface active compounds as promising strategy to enhance oil biodegradability, inoculation with biosurfactant-producing microorganisms has also been considered as an interesting methodology. This practice would offer the advantage of a continuous supply of a non-toxic and biodegradable surfactant at a low cost. Also, the use of microbes having co-existing capacity to degrade hydrocarbons and produce biosurfactants can effectively be used for speedy bioremediation of oil-contaminated sites (Kumar et al. 2006). In fact, hydrocarbon-degrading bacteria are well known by their ability to produce biosurfactants in situ facilitating therefore their microbial life in environments dominated by hydrophobic compounds (Kumar et al. 2007; Ganesh and Lin 2009; Reddy et al. 2010; Mnif et al. 2011).

In this context, the present work has focused on this approach, aiming to ameliorate diesel biodegradation using a selected consortium by the addition of biological emulsifier and/or co-inoculation with biosurfactant-producing microorganisms. The used consortium was a combination of three newly isolated strains. Two other strains, able to degrade hydrocarbon and produce a bioemulsifier, were used as an in situ biosurfactant-producing strains. An exogenously lipopeptide biosurfactant has been also added. Studies were conducted in order to assess the process feasibility and compare the effectiveness of the different strategies used.

Materials and methods

Strain isolation, characterization, and screening for hydrocarbon degradation

Microbial strains having the ability to degrade hydrocarbon were isolated from hydrocarbon-contaminated soil by the enrichment culture technique. Ten grams of soil were added to 50 ml of minimal-salt medium supplemented with 5 % (*v*/*v*) of diesel oil and were incubated at 37 °C at 150 rpm. Three cycles of enrichment were made until complete disappearance of the oil phase. At the end of incubation, serial dilutions (1/10) from the enrichment culture were plated out into mineral salt agar plates coated with diesel oil on their surface and incubated under aerobic conditions at 37 °C until colony formation. Isolated strains were purified by plate streaking method on LB agar medium until having pure isolated colonies. The purity of the isolates was checked microscopically. Strains were stored on LB agar medium at 4 °C until further use. For long-term preservation, the bacterial isolates were stored in 30 % glycerol at −70 °C (Ghribi et al. 2012).

Initially, the newly isolated strains were screened for their growth on liquid mineral salt medium supplemented with diesel oil. Degradation of diesel oil was evaluated by the gravimetric method in the end of cultivation. The strains having the best growth with better diesel biodegradation were subjected to further characterization.

Identification of the newly isolated bacteria and phylogenetic analysis

The bacterial isolates were identified by sequence analysis of nearly complete 16S rRNA gene and phylogenetic analysis. To proceed, the genomic DNA was extracted using the Pure Link Genomic DNA extraction kit, following the manufacturer's recommended procedure. The 16S rDNA genes were

amplified using Rd1 (5′AAGGAGGTGATCCAAGCC3′) and Fd1 (5′AGAGTTTGATCCTGGCTCAG3′) universal primers and a "Gene Amp PCR System 2700" (Applied Biosystems). The 25 μl of PCR mixture contained the following: 0.2 mM of deoxynucleoside triphosphate, 1.32 μM concentration each primer, 0.5 U of DNA polymerase, 5 μl of ×5 buffer, and 1 μl (5 ng) of total DNA template. The PCR reaction conditions included initial denaturation at 94 °C for 5 min followed by 35 cycles including a denaturation step at 94 °C for 1 min, annealing step at 60 °C for 1 min, and extension step at 72 °C for 2 min, and a final extension for 10 min at 72 °C was performed. The purified PCR products (with Invitrogen Gel Extraction Kit, by Life Technologies) were sequenced in both directions using an automated sequencer by Applied Biosystems (USA). Processed sequences were subjected to BLAST (Basic Local Alignment Search Tool) alignment. The MEGA program version 6.06 was used to make multiple alignments between all sequences using default parameters (Thompson et al. 1994).

Preparation of inoculum

The inoculum was prepared in LB medium as described by Mnif et al. (2012). The resultant culture was centrifuged for 10 min at 10,000 rpm, and the cell pellet was washed twice in sterile saline solution (9‰) before inoculation (Ganesh and Lin 2009). For the different inoculation conditions, inoculates were mixed at equal proportion and the resultant mixture served for inoculation to start with an optical density of 0.1 (Mnif et al. 2012).

Culture conditions and biodegradation assays

Diesel biodegradation assays were performed in liquid mineral medium as well as in soil amended with hydrocarbons to assess the effect of biosurfactants on diesel solubility and biodegradation. The liquid media composition used for biodegradation study was as described in our previous work with slight modifications (Mnif et al. 2012). It contains (g/l) $(NH_4)_2SO_4$ (1), K_2HPO_4 (1), KH_2PO_4 (0.5), $MgSO_4$ (0.2), NaCl (0.5), $MnSO_4$ (0.001), $FeSO_4$ (0.001), $ZnSO_4$ (0.001), and $CaCl_2$ (0.001). For the treatment of diesel-contaminated water, biodegradation assays were performed in 250-ml Erlenmeyer flasks containing 50 ml of liquid mineral medium supplemented with 5 % diesel (*v*/*v*). Un-inoculated control flasks with diesel were incubated in parallel to monitor abiotic losses of the substrate. The culture flasks were incubated for 21 days at 37 °C under shaking at 150 rpm. The growth patterns were obtained by measuring optical density at 600 nm at defined interval times. All experiments were performed in triplicate.

Determination of diesel degradation

The level of diesel degradation was determined using the gravimetric analysis method (Ganesh and Lin 2009), and residual hydrocarbons were analyzed by gas chromatography after 3 weeks of incubation. For liquid medium, the culture broth (50 ml) and abiotic control were collected, centrifuged at 10, 000 rpm for 10 min, and the resultant supernatant was extracted with chloroform (*v*/*v*) to recover the residual hydrocarbon.

The organic fraction was evaporated by the rotary evaporator system and the remaining hydrocarbon was weighted after desiccation at 60 °C. The percentage of degraded hydrocarbon was calculated as proposed by Ganesh and Lin (2009):

$$\text{Hydrocarbon degradation}(\%) = \left[\frac{\left(\begin{array}{c}\text{weight of residual hydrocarbon in the abiotic control}-\\ \text{weight of residual hydrocarbon in the assay sample}\end{array}\right)}{\text{original weight of deisel introduced}}\right]\times 100$$

Improvement percentages of oil biodegradation were calculated according to the following formula as proposed by Mnif et al. (2014):

$$\text{Biodegradation improvement}(\%) = \left[\frac{\left(\begin{array}{c}\text{hydrocarbon degradation in the presence of the bioemulsifier or the biosurfactant producing strain}-\\ \text{hydrocarbon degradation with the consortium alone}\end{array}\right)}{\text{hydrocarbon degradation with the consortium alone}}\right]\times 100$$

Gas chromatography analyses

Gas chromatography analyses were done according to Mnif et al. (2014). Residual hydrocarbons were extracted using an organic solvent as described in the previous section. The organic solvent extract containing the residual hydrocarbons was subjected to gas chromatography analysis (Shimadzu Gas Chromatograph system, GC-2014). The system was also

equipped with a flame ionization detector and a 60-m fused silica capillary column (DB5, Agilent J&W). The injector and detector temperatures were both set at 320 °C with 5 ml/min of hydrogen as the carrier gas. The column temperature was initially set at 100 °C, then raised to 320 °C at the rate of 5 °C/min, and finally set at 320 °C for 10 min (Mnif et al. 2014).

Results and discussions

Isolation and identification of diesel-degrading bacteria

Diesel is a high-diffuse contaminant. Bio-restoration of diesel-contaminated sites requires the involvement of microorganisms having the capability to survive on highly contaminated sites and assimilate diesel. Hence, strain screening was based on the ability of diesel degradation suggesting the utilization of this hydrocarbon as sole carbon and energy source. In fact, after three cycles of enrichment on basal minimal medium containing 5 % (*v*/*v*) diesel, the medium became turbid indicating microbial growth. Then, a total of 25 hydrocarbon-utilizing microorganisms were isolated from the contaminated soil after the enrichment process.

The efficiency of bioremediation is often a function of the microbial population or consortium and how it can be enriched and maintained in an environment. Inoculation of bacteria with hydrocarbon biodegradation capabilities shortens the time of the treatment. In fact, the mixed bacterial consortium showed more growth and degradation than did individual strains. Therefore, we selected the most interesting strains, among the isolates, in view of their abilities to grow and degrade diesel oil for further studies. The molecular identification and phylogenetic analysis showed that they correspond to *Lysinibacillus bronitolerans* RI18 (KF964487), *Bacillus thuringiensis* RI16 (KM111604), and *Bacillus weihenstephanensis* strain RI12 (KM094930). A fourth strain, *Acinetobacter radioresistens* RI7 (KJ829530), having the ability to degrade hydrocarbon and produce biosurfactant was also isolated. The three first strains constituted the consortium, and the fourth strain was used for the co-inoculation by a biosurfactant-producing strain as well as *Bacillus subtilis* SPB1 (HQ392822) (Ghribi et al. 2012).

According to literature studies, Gram-negative hydrocarbon-tolerant bacteria belong generally to *Acinetobacter*, *Pseudomonas*, *Alkanivorax*, and related genera (Yakimov et al. 2005; Ganesh and Lin 2009). They are generally associated with a fast petroleum degradation phase and are the more efficient (Ganesh and Lin 2009). Gram-positive bacteria such as *Bacillus*, *Rhodococcus*, *Staphylococcus*, and *Exiguobacterium* have also been found to be hydrocarbon-tolerant bacteria (Lazaroaie 2010).

B. thuringiensis was widely described as a hydrocarbon-degrading bacteria (Maiti et al. 2012; Thamer et al. 2013; Archaya et al. 2014). *Lysinibacillus* sp. was also published in many previous studies as potent hydrocarbon-degrading microorganisms (Mnif et al. 2011; Ben Hamed et al. 2013). Also, *Lysinibacillus sphaericus* was reported as potent dibenzothiophene and cyfluthrin-degrading bacteria as described by Bahuguna et al. (2011) and Hu et al. (2014). However, until now, hydrocarbon biodegradation by *B. weihenstephanensis* strains was not reported by any study. Also, *A. radioresistens* was widely described as hydrocarbon-degrading (Dehghani et al. 2013) and biosurfactant-producing strain (Barkay et al. 1999; Toren et al. 2001).

Growth kinetics of the selected consortium under different cultural conditions

Regarding literature reports, bacterial consortia were highly used to treat hydrocarbon-contaminated soil and water. In fact, mixed cultures were demonstrated more efficiently than pure culture in hydrocarbon decontamination (Adebusoye et al. 2007; Sathishkumar et al. 2008; Liu and Liu 2011). Since, bacterial degradation could be limited by the low solubility and bioavailability of the target hydrocarbons, diesel biodegradation was studied by the selected consortium in the presence and the absence of the *B. subtilis* SPB1 biosurfactant, in one hand, and when co-inoculating by biosurfactant-producing strains, *B. subtilis* SPB1, and a newly isolated strain *A. radioresistens* RI7, in another hand. In fact, five culture conditions were assayed as follows: consortium alone, consortium+SPB1 biosurfactant, consortium+*B. subtilis* SPB1 strain, consortium+*A. radioresistens* RI7, and consortium+*A. radioresistens* RI7+*B. subtilis* SPB1. The used SPB1 biosurfactant was produced and prepared according to the protocol described in our previous work (Mnif et al. 2013a).

In this purpose, kinetics of growth of the microbial consortium on diesel oil was followed by measuring the evolution of optical density at 600 nm (Fig. 1). Growth at the expense of diesel was verified by demonstrating an increase in the optical density at 600 nm, suggesting a bacterial growth. The growth of the consortium composed of the three strains (*L. bronitolerans* RI18; *B. thuringiensis* RI16; *B. weihenstephanensis* RI12) with diesel as a unique carbon source indicates that these microorganisms are potential diesel bio-degraders.

For liquid experiments, after a certain time of incubation, bacterial growth rate becomes stable and the stationary phase was reached (Fig. 1). This is due to the fact that the increasing numbers of bacteria began to compete for the dwindling nutrients and their exponential growth was inhibited. Also, when using hydrocarbons as a unique source of carbon and energy, a short-term stationary phase was observed, suggesting a rapidly diminishing rate of nutrient availability essential to

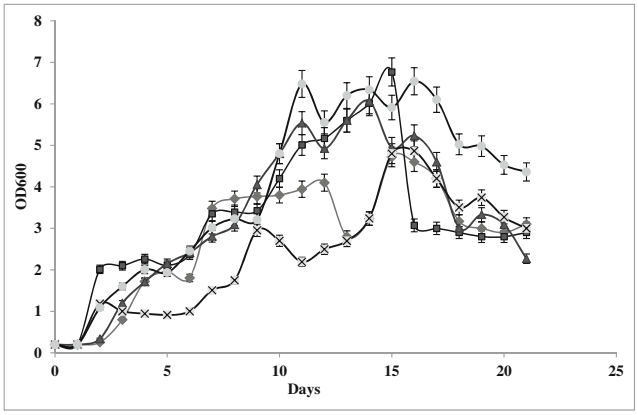

Fig. 1 Growth profiles of the selected consortium alone, with SPB1 biosurfactant, with *B. subtilis* SPB1, with *A. radioresistens* RI7, and with *A. radioresistens* RI7 and *B. subtilis* SPB1 in liquid medium supplemented with 5 % diesel oil (*v*/*v*)

maintain live culture. At the end of the third week of incubation, a gradual decrease of the bacterial growth was observed, suggesting the initiation of the death phase. It was assumed that detrimental environmental changes, such as nutrient deprivation and the buildup of toxic wastes, caused irreparable harm and loss of viability (Mnif et al. 2014). This was verified by the fact that, even when bacterial cells were transferred to a fresh medium, no cellular growth was observed (Mnif et al. 2014).

Distinct amelioration of bacterial consortium growth was recorded when adding biological emulsifiers or when co-cultured with biosurfactant-producing bacteria. The enhancement of bacterial growth could be owed to a better assimilation of diesel oil as a unique source of carbon and energy. Also, biosurfactant addition seemed to accelerate the first growth phase that can be due to the assimilation of emulsifier as an easier carbon source for the initiation of growth stimulating after that growth on immiscible substrate. Furthermore, the enhancement of growth was more pronounced with co-culture with *B. subtilis* SPB1 or *A. radioresistens* RI7.

Estimation of diesel biodegradation potency and gas chromatography analyses of residual hydrocarbons

In order to determine the biodegradation rate of diesel, the residual oil content was determined using the gravimetric analysis method (Ganesh and Lin 2009). Results of Table 1 confirmed that the addition of the biological emulsifier supported an enhancement of hydrocarbon degradation. In fact, SPB1 lipopeptide biosurfactant supplement hold up an improvement of about 38.42 %. The comparison of the enhancement of oil biodegradation due to the addition of lipopeptide bioemulsifier with those of co-culture with biosurfactant-producing strain (*A. radioresistens* RI7 and/or *B. subtilis* SPB1) was done. Results were better when culturing *B. subtilis* SPB1 strain with the respective consortium with an improvement of about 49.65 %. Furthermore, the best improvement evaluated to be about 55.4 % was recorded for the consortium cultured with *B. subtilis* SPB1 and *A. radioresistens* RI7 strains. The results demonstrated that bacteria with the catabolic potential to degrade hydrophobic contaminants could interact synergistically with surfactant-producing strains to hasten the biodegradation of diesel. In fact, the biosurfactant produced in situ can contribute significantly to the emulsification and solubilization of diesel oil increasing therefore its bioavailability for hydrocarbon-degrading strain. Moreover, the biosurfactant-producing strains can participate in the biodegradation process enhancing diesel digestion.

To confirm the obtained results, gas chromatographic analyses of the residual hydrocarbons were carried out after 21 days of aerobic incubation. In fact, regarding previous literature studies, gas chromatography has been widely applied in order to assess hydrocarbon degradation (Marquez-Rocha et al. 2001; Abalos et al. 2004; Chamkha et al. 2011; Mnif et al. 2014). The obtained chromatograms are presented in Fig. 2. They confirmed the described results and suggested that the present consortium was highly effective on different diesel compounds. Assays were compared with abiotic control, carried out at the same conditions. In fact, gas chromatography profiles showed a decrease of the peak intensity of each compound. The appearance or disappearance of novel peak probably resulted either from the degradation of the compounds or the synthesis of novel metabolites in the fermentation media. Also, the improvement of diesel degradability due to the different combination realized was confirmed by gas chromatography analysis.

Biodegradability of hydrocarbons and hence their degree of persistence in natural environments are influenced by various factors, most vital of which are the chemical structures of the hydrocarbons, the presence of viable microbial population able to degrade them, and the environmental conditions optimal for microbial biodegradation activities. Diesel oil derives from the distillation of crude oil, having a complex hydrocarbon combination with carbon numbers that range approximately from C9 to C20, iso-alcanes, cyclo-alcanes, paraffin, olefins, naphtha, and aromatic compounds (Yakimov et al. 2005). This oil mixture represents an excellent substrate in the study of hydrocarbon biodegradation due to the complexity of its composition (Mnif et al. 2014).

The use of bacterial consortia able to degrade hydrocarbons knows a great interest. It is generally considered that mixed cultures have a higher metabolic versatility than pure cultures. Previous studies reported the selection of diverse bacterial consortia for the bioremediation of hydrocarbons; *Pseudomonas* sp. BPS1-8, *Bacillus* sp. IOS1-7, *Pseudomonas* sp. HPS2-5, and *Corynebacterium* sp. BPS2-6 described by Sathishkumar et al. (2008); *Achromobacter*, *Alcaligenes*, and *Cupriavidus* described by Bacosa et al. (2010); and *Acinetobacter faecalis* WD2, *Staphylococcus* sp. DD3, and

Table 1 Biodegradation of diesel by the selected consortia in liquid media; effect of juin biosurfactants addition and co-inoculation with biosurfactant-producing strains

	Cultural condition	Hydrocarbon biodegradation (%)	Biodegradation improvement (%)
Diesel-contaminated water (5 % *v*/*v*)	Consortium alone	$31.44^{a}\pm1.640$	–
	Consortium+SPB1 BioS	$43.52^{b,\ a}\pm1.520$	38.42
	Consortium+SPB1	$47.05^{c,\ a}\pm1.650$	49.65
	Consortium+RI7	$37.71^{d,\ b}\pm1.510$	19.94
	Consortium+SPB1+RI7	$48.86^{e,\ c}\pm1.560$	55.4

Cells were cultivated in basal medium supplemented with 5 % (*v*/*v*) diesel. Mean scores in the same column with different letter are significantly different (for $p<0.05$), the first letter designates the significance between the control test (consortium alone) and each of the parameters, and the second letter designates the mutual significance between the different parameters. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test

SPB1 BioS SPB1 biosurfactant, *SPB1 B. subtilis* SPB1 strain*RI7 A. radioresistens* RI7 strain

Neisseria elongate TDA4 described by Mukred et al. (2008). Also, Owsianiak et al. (2009) and Marquez-Rocha et al. (2001) reported the biodegradation of diesel oil by microbial consortia. Our present study reported the use of a mixed culture. Certainly, the reduced solubility of hydrocarbons in aqueous media can limit their bioavailability and

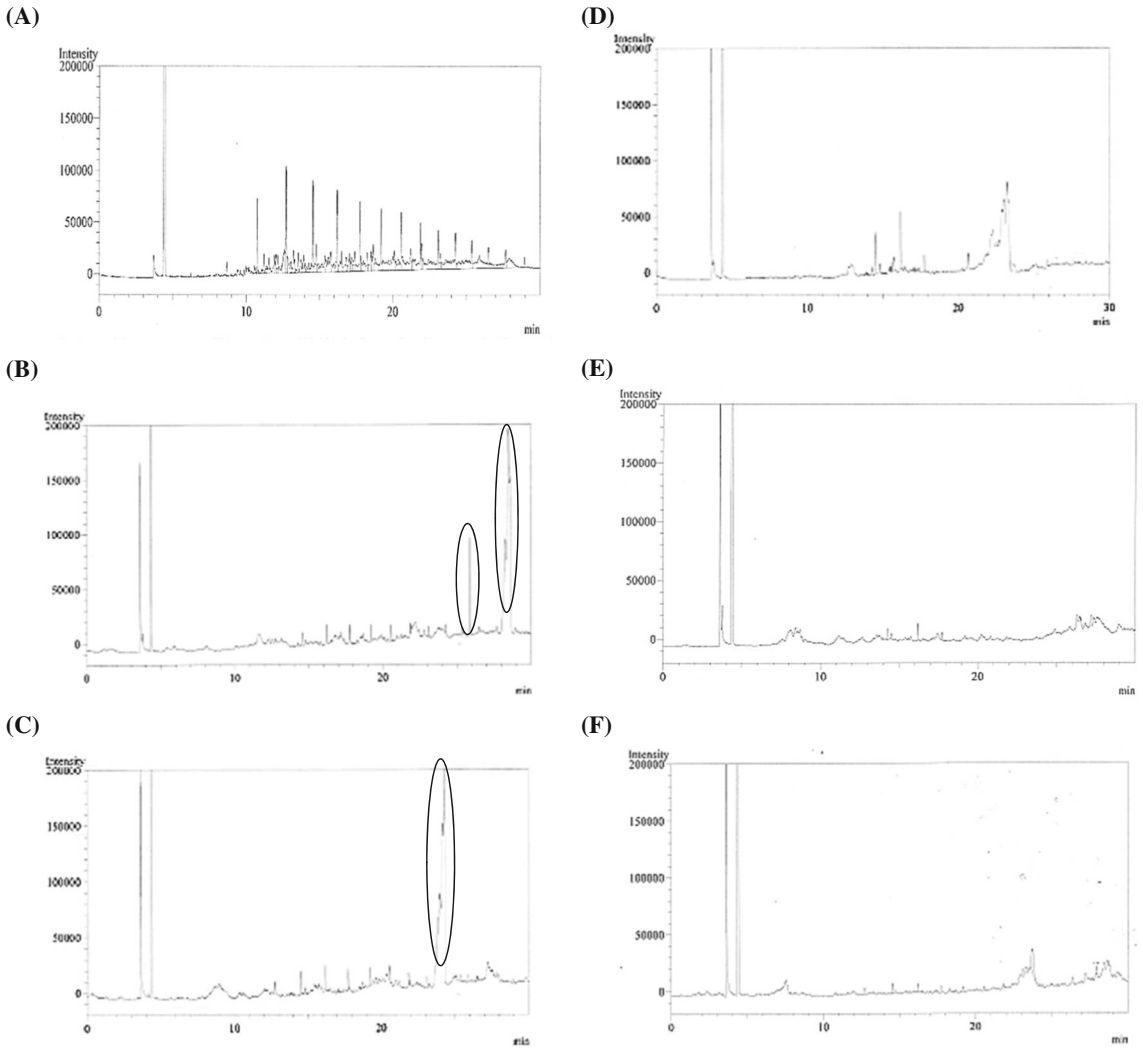

Fig. 2 Gas chromatography profiles of diesel oil remaining in the mineral salt medium in liquid experiments after incubation without SPB1 biosurfactant (**b**), with SPB1 biosurfactant (**c**), with *B. subtilis* SPB1 (**d**), with *A. radioresistens* RI7 (**e**), and with *B. subtilis* SPB1+ *A. radioresistens* RI7 (**f**). The first spectrum (**a**) shows a negative control prepared in the same conditions but without inoculation

biodegradation. Certain strains of *B. subtilis* were able to produce biosurfactants (Bacosa et al. 2010; Chander et al. 2012; Ghribi et al. 2012; Santos et al. 2014) and degrade hydrocarbon (Lily et al. 2012; Yuliani et al. 2012). Other *B. subtilis* strains are well known by their ability to produce biosurfactant simultaneously with hydrocarbon degradation for enhancing hydrocarbon bioavaibility and uptake (Wijanarko et al. 2012; Gudina et al. 2013; Yengejeh et al. 2014). In this aim, *B. subtilis* SPB1, a biosurfactant-producing strain and demonstrated in our previous work to have the ability to degrade hydrocarbons (Mnif et al. 2014), was used as an indigenous biosurfactant-producing strain in order to increase the consortium efficiency. Furthermore, addition of biosurfactants alone can enhance the process feasibility. In fact, biosurfactants can be used as alternatives of synthetic emulsifiers to enhance hydrocarbon biodegradation by the enhancement of mobilization, solubilization, or emulsification of hydrophobic compounds (Pacwa-Plociniczak et al. 2011). Generally, surfactants enhance bioremediation by increasing the surface area of hydrophobic substrates available for biodegradation and increase the bioavailability of these substrates (Pacwa-Plociniczak et al. 2011). Moreover, our recent study confirmed that SPB1 biosurfactant addition enhances remarkably diesel solubilization in water promoting its emulsification (Mnif et al. 2013b). These can predict its potential role on the enhancement of diesel bioavailability for hydrocarbon bacterial-degrading flora, stimulating then its assimilation. So, SPB1 bioemulsifier was added in order to enhance the diesel biodegradation.

The obtained results showed the enhancement of diesel biodegradation in all the studies cases. In fact, either biosurfactant added alone or that produced in situ acts to enhance diesel oil removal. Moreover, SPB1 bioemulsifiers added alone can be of great interest as it ensured alone about 70 % of the whole improvement when compared with co-culture of the two biosurfactant-producing strain. But, for field bioremediation application based on bioaugmentation, addition of the biosurfactant-producing bacteria may be beneficial and more practical than exogenously adding purified biosurfactant (Kumar et al. 2006). This could avoid the high cost of production and preparation of biosurfactant and facilitate the process conduction. However, the effect of in situ biosurfactant production by co-culture of the biosurfactant-producing and hydrocarbon-degrading bacteria on hydrocarbon degradation is relatively less studied than the supplement by biosurfactant alone (Lai et al. 2009). Furthermore, in all experiments, SPB1 biosurfactants addition seemed not to have a deleterious effect on growth patterns, in contrary; it seems to induce consortium growth and diesel degradation probably through its solubilization. Literature reviews discussed the enhancement of hydrocarbon solubility in the presence of microbially derived surfactants (Ji et al. 2009; Abouseoud et al. 2010; Pacwa-Plociniczak et al. 2011).

To date, many literature studies reported the enhancement of hydrocarbon assimilation in liquid media by microbial strains due to the addition of biological emulsifiers. *P. aeruginosa*-derived rhamnolipid and *B. subtilis*-derived surfactin were demonstrated by Whang et al. (2008) to increase microbial growth and diesel biodegradation. In fact, they significantly enhanced biomass growth and improved diesel biodegradation from 40 to 94 % when adding 40 mg/L of surfactin and from 40 to 100 % when adding 80 mg/L of rhamnolipid. Moran et al. (2000) reported the augmentation of hydrocarbon digestion by *B. subtilis* O9 surfactin supplement. Moreover, adding of rhamnolipid produced by *P. aeruginosa* AT10 improved significantly biodegradation potency of crude oil, by a bacterial consortium, from 32 to 61 % after 10 days of incubation (Maiti et al. 2012). Rhamnolipid biosurfactants were also demonstrated by Owsianiak et al. (2009) as enhancers of diesel, biodiesel, and gasoline degradation. *Candida antarctica* T-34-derived biosurfactants were shown to improve the biodegradation rate of some *n*-alkanes, a mixture of *n*-alkanes and kerosene (Hua et al. 2004). At the same way, it was demonstrated by Saeki et al. (2009) that *Gordonia* sp. JE-1058 biosurfactant addition stimulated crude petroleum biodegradation by indigene marine flora.

Another hypothesis proposes that the enhancement of bacterial growth and therefore hydrocarbon degradation when adding biosurfactant could be due to the fact that this biosurfactant is biodegradable and could be used as a primary and easier carbon source to initiate microorganisms' growth on water-immiscible substrates (Bautista et al. 2009).

Otherwise, in addition to the reduced bioavailability and solubility of the pollutants recognized as substrates, biodegradation ability of bacteria is assumed to be associated with the production of different enzymes and their potent activity. Accordingly, we can assume the beneficial effect of the emulsifiers on diesel biodegradation through the increase of bacterial membrane permeability facilitating then substrates diffusion and/or metabolites and enzymes secretion. Many previous reports described the same findings for surfactants (Ji et al. 2009; Gül and Dönmez 2012; Hadibarata et al. 2013) or biosurfactants (Jadhav et al. 2011; Zhou et al. 2011). Moreover, as described in many other researches, surfactants could promote enzymes activities (Kristensen et al. 2007; Champagne et al. 2010; Zhang et al. 2012).

Conclusion

An effective diesel-degrading consortium was isolated from Tunisian contaminated soil. Diesel biodegradation was studied by the selected consortium under different cultural conditions. The consortium was composed with bacterial strains belonged to *Bacilus* and *Acinetobacter* genera. Various conditions were studied in order to improve the capacity of diesel

oil degradation by this consortium. Among them, we found out the co-inoculation with biosurfacatant-producing bacteria *B. subtilis* and *A. radioresistens* increased the degradation for more than 50 %. The direct addition of crude biosurfactant improves also the yield of degradation to about 38.42 %. Gas chromatography analyses are correlated with the gravimetric evaluation of the residual hydrocarbons. Our finding suggested the potential applicability of the selected consortium along with the ex situ- and in situ-added biosurfactant for the bioremediation of diesel-contaminated water.

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Ethical statement The experimental protocols were conducted in accordance with the Guide for the Care and Use of Laboratory Animals issued by the University of Sfax, Tunisia, and approved by the Tunisia Committee of Animal Ethics.

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