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ORIGINAL PAPER

Bio-emulsifying and biodegradation activities of syringafactin producing *Pseudomonas* spp. strains isolated from oil contaminated soils

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Abstract *Pseudomonas* strains isolated from oil contaminated soils were screened for biosurfactant production. Three out of eleven *Pseudomonas* isolates were selected for their high emulsifying activity (E24 value on *n*-hexadecane ~ 78%). These isolates (E39, E311 and E313) were identified as members of the *P. putida* group using phenotypical methods and a molecular approach. To identify the chemical nature of produced biosurfactants, thin layer chromatography and MALDI-ToF mass spectrometry analysis were carried out and revealed lipopeptides belonging to the syringafactin family. The activity of the produced biosurfactants was stable over a pH range of 6–12, at high salinity (10%) and after heating at 80 °C. Tests in contaminated sand micro-bioreactors showed that the three strains were able to degrade diesel. These results suggest the potential of these syringafactin producing strains for application in hydrocarbon bioremediation.

Keywords *Pseudomonas* · Biosurfactant · Bioemulsifier · Biodegradation · Lipopeptide

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Introduction

Surfactants are amphiphilic chemical compounds, i.e. they contain both hydrophobic and hydrophilic groups, that accumulate preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. They are used industrially as emulsifiers, wetting, dispersing and foaming agents. Microbial surfactants, known as biosurfactants, are produced by diverse microorganisms such as bacteria, fungi and yeasts, and are structurally diverse (Desai and Banat 1997; Karanth et al. 1999; Lang 2002; Mulligan 2005). They are grouped into low molecular weight compounds including glycolipids, lipopeptides and phospholipids, and into high molecular weight polymers including proteins, lipoproteins, polysaccharides and lipopolysaccharides (Ron and Rosenberg 2002).

In comparison to synthetic surfactants, biosurfactants offer several advantages mainly due to their high

biodegradability, which makes them more eco-friendly. Furthermore, some of these molecules have a good stability on extreme conditions of pH, temperature and salinity (Marchant and Banat 2012). These properties render them appropriate for many application fields such as polluted soils bioremediation (Cameotra and Makkar 2010; Pacwa-Płociniczak et al. 2011), agriculture (Sachdev and Cameotra 2013), food industry (Campos et al. 2013), and animal and human health (Cameotra and Makkar 2004; Rodrigues et al. 2006).

Pseudomonas spp. are well-known for their ability to produce biosurfactants mainly rhamnolipids and lipopeptides (Mulligan 2005). Rhamnolipids, glycolipids which consist of one or two molecules of rhamnose linked to one, two or three molecules of β-hydroxyfatty acid (Abdel-Mawgoud et al. 2010), are some of the best studied biosurfactants. Lipopeptides are powerful biosurfactants that are composed of a lipid tail linked to a short linear or cyclic oligopeptide. They are structurally very diverse, depending on the configuration, type and number of the amino acids in the peptide and on the composition and length of the fatty acid. They are divided into several families: the majors are viscosin, amphisin, orfamide, putisolvin, tolaasin, entolysin, syringopeptin, syringomycin and syringafactin families (Raaijmakers et al. 2010; Roongsawang et al. 2011). Rhamnolipids and lipopeptides are implicated in many important bacterial functions, including antimicrobial activity, swarming motility, attachment, biofilm formation and effect on bioavailability allowing the solubilisation of insoluble compounds by increasing wettability and emulsification of water. Biosurfactants can also protect cells from toxic compounds and heavy metals by encapsulating them into micelles (Ron and Rosenberg 2001; Van Hamme et al. 2006). Biosurfactant producing *Pseudomonas* are commonly found in different environments including soil and the rhizosphere (Fechtner et al. 2011), particularly in hydrocarbon-contaminated soils where they facilitate hydrocarbon degradation (Bodour et al. 2003; Bento et al. 2005; D'aes et al. 2010; Saikia et al. 2012; Mohammed et al. 2015) and on plants where they can be beneficial or harmful for their host (D'aes et al. 2010). For these reasons, *Pseudomonas* are good candidates for discovering new biosurfactants. The present study aimed to screen *Pseudomonas* spp. strains isolated from hydrocarbon contaminated soil for their potential to produce biosurfactants/bioemulsifers. The most potent isolates were tested for application in diesel biodegradation.

Materials and methods

Isolation of *Pseudomonas* strains

Bacterial colonies were isolated from an oil-contaminated soil from an auto-mechanical workshop, in the area of Sfax, Tunisia. 500 g of soil samples were collected from the surface, at 5 and 10 cm of depth. 1 g of each sample was mixed with 10 mL physiological water by vortexing for 10 min; then several dilutions were prepared on physiological water, spread on an LB agar medium and incubated at 30 °C for 24 h. Colonies were selected on the basis of colony morphology, transferred separately on cetrimide agar a selective medium for *Pseudomonas* isolation, and incubated at 30 °C for 24 h. *Pseudomonas* bacteria were preserved on cryotubes containing 40% of glycerol solution and stored at − 20 °C for short preservation and − 80 °C for long preservation.

Bacterial growth conditions and cell-free supernatant preparation

Colony growth was performed on Tryptic Soy Agar plates streaked with frozen stocked cells at a temperature of 30 °C for 24 h. Liquid culture was performed in Tryptic Soy Broth (pancreatic digest of casein 17 g L^{-1}; peptic digest of soybean meal 3 g L^{-1}; D-glucose 2.5 g L^{-1}; sodium chloride 5 g L^{-1}; dipotassium hydrogen phosphate 2.5 g L^{-1}) at 30 °C for 72 h with shaking on 160 rpm. A 10 mL preculture was inoculated with an isolated colony after 24 h of incubation. Then, 0.1 mL of the 24 h preculture served to inoculate a 100 mL culture which was incubated in the same conditions for 48 h. Broth culture was centrifuged for 10 min at 10,000×*g*. The obtained supernatant was sterilized through a 0.22 µm Millipore PES filter membrane and stored at 4 °C for biological tests.

Preparation of crude extract of biosurfactants

Biosurfactants were precipitated overnight at 4 °C by adding HCl (6 M) to the free cell supernatant to a pH of 2 (Cooper and Goldenberg 1987; Peypoux et al.

1999). The mixture was centrifuged at 10,000×*g* for 20 min and then the pellet was resuspended in ultrapure water. Finally, the pH was adjusted to 7 with NaOH (1 M) and the crude extract was lyophilized using a Heto Power Dry PL 9000 freeze-dryer (Jouan Nordic, Allerod, Denmark), according to the following steps: 1 h at − 30 °C, 5 h at − 10 °C, 5 h at 0 °C, 5 h at 20 °C, 5 h at 35 °C. Freeze-drying was carried out under a residual pressure of 15 mbar. For utilisation, lyophilized crude extract was then solubilized in ultrapure water so that to obtain the adequate concentrations (1% or 0.1% w/v) and stored at 4 °C.

Screening for biosurfactant production

Drop collapse test

To identify biosurfactant-producing strains, a drop collapsing test was carried out according to Bodour and Miller-Maier (1998). 2 µL of paraffin oil was added to each well of a 96-well microliter plate and allowed to equilibrate for 1 h at room temperature. Then, 5 µL of cell-free supernatant of *Pseudomonas* spp. isolates were added on oil surface. The result was observed after 1 min. The cultures which gave flat drops were scored as positive, while the cultures that gave rounded drops which pointed-out a deficiency of biosurfactant production were scored as negative.

Surface tension and F_{CMC} measurement

The surface tension was measured on cell-free supernatants and on the crude extracts of *Pseudomonas* spp. isolates using a tensiometer (TD1 LAUDA, Lauda-Königshofen, Germany) according to the Du Noüy ring method. Pure water was used as control, giving a surface tension value of 72 mN m^{-1}. Critical micelle concentration (CMC) is specified as the concentration at which the micelle formation is initiated in the solution containing the amphiphilic compound. CMC represents the intersection of linear component of the curve drawn between the surface tension and the biosurfactant concentration. In our case, surface tension measurements were performed on seven different dilutions of supernatants in ultrapure water: 1/20; 1/10; 1/5; 1/4 1/2; 1 at room temperature which provides the dilution of the supernatant at which the CMC is reached (Chakraborty et al. 2011). The F_{CMC} value is determined as an indirect estimation of the CMC by the determination of dilution value where there was a sharp rise in the surface tension (Cooper and Zajic 1980).

Oil displacement test

This assay was performed according to Morikawa et al. (2000). 40 mL of distilled water were placed on the surface of a large Petri dish with 150 mm in diameter, followed by adding 15 µL of crude oil (diesel) on the water surface, and 20 µL of the isolate culture supernatants on the centre of the oil film. Then, the diameter of the clear halo visualized under visible light was measured after 30 s. If biosurfactants are present, the oil will be displaced resulting in an oil free clearing zone. A larger diameter of oil depicts a higher activity of biosurfactant.

Emulsion index determination

Emulsifying activity was measured according to the Cooper and Goldenberg (1987) method. 4 mL of biosurfactant crude extract were mixed with 4 mL of *n*-hexadecane and vortexed at high speed for 2 min. The mixture was allowed to stand for 24 h before measurement and the emulsion index was defined as follows:

$$E24(\%) = \frac{height\ of\ emulsion\ layer}{total\ height} \times 100$$

Identification of bacterial strains

To characterise the phenotypic properties of isolated strains, tests including morphological characterisation, Gram staining, oxidase activity and carbohydrate fermentation profile determined by API 20 NE tests (Biomerieux; Marcy l'Etoile, France) were performed. Each fermentation profile was processed by the APIWEB software database for identification. Genotypic characterisation was performed as follows. Genomic DNA was isolated from a one-night bacterial culture using the Wizard Genomic Purification DNA Kit (Promega Corp., Madison, WI, USA) following the prescribed standard protocol.). The whole 16S rRNA gene was amplified by polymerase chain reaction (PCR) using primers S1 (5-AGAGTTTGAT C(A,C)TGGCTCAG-3) and S2 (5-GG(A,C)TACCTT

GTTACGA(T,C)TTC-3). The PCR reaction was performed with the following mixture: 5 µL of extracted genomic DNA (200 ng μL^{-1}), 2.5 µL of each primer (20 mM), 25 µL of PCR Master Mix (Thermo Fisher Scientific Fermentas, Vilnius, Lithuania) and ddH2O were mixed up to a final volume of 50 µL. PCR thermal cycling was carried out using a thermal cycler as follows: a first denaturation of the DNA at 94 °C for 3 min, than 30 cycles of denaturation at 94 °C for 40 s, primer annealing at 55 °C for 50 s, and primer synthesis step at 72 °C for 12 min. The PCR products were subjected to agarose gel electrophoresis and purified using a QIA quick DNA gel purification kit (Qiagen, Hilden, Germany). Then, purified PCRs were introduced into *Escherichia coli* JM109 using the pGEMT Easy vector kit (Promega Corp). Transformed cells were selected on Luria–Bertani (LB) solid medium supplemented with 100 g L^{-1} of ampicillin. Plasmids were isolated from transformant colonies using QIAprep plasmid extraction kit protocol (Qiagen). Restriction endonuclease analysis of the plasmids was carried-out using EcoRI (Thermo Fisher Scientific Fermentas). Cloned products were sequenced and analysed as above. The sequencing of resulting cloned product was performed by the Custom Sequencing Service of Eurofins Genomics (Ebersberg, Germany) based on cycle sequencing technology on ABI PRISM 3730XL. Two sequencing reactions generating both forward and reverse sequences between 900 and 1000 pb were required to cover the length of the 16S rRNA gene (about 1500 pb). Sequences were trimmed and assembled as previously described (El Arbi et al. 2016). The 16S rRNA gene sequences obtained from the isolate E39, E311 and E313 were compared with other bacterial sequences using NCBI megaBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. Then, a maximum-likelihood phylogenetic tree was built with PhymL on SeaView version 4. All sequences were submitted to the GenBank database [accession numbers: MG183696 (E39), MG183695 (E311), and MG188738 (E313)].

Thin layer chromatography

The type of biosurfactant was preliminary determined by using a thin layer chromatography (TLC) analysis. A volume of 40 µL of crude biosurfactant extracted from E39, E311 and E313 strains supernatant was spotted into three silica gel plates (F-254; Fisher) using CAMAG automatic TLC sampler. Samples were developed using $CHCl_3$: CH_3OH: H_2O (65:15:2 v/v/v) as a mobile phase. Then the plates were air-dried, sprayed with standard reagents and heated at 105 °C during 5 min. Ninhydrin reagent (0.5 g ninhydrin + 100 mL acetone) was used for peptide detection and anthrone reagent (1 g anthrone + 5 mL sulphuric acid + 95 mL ethanol) for sugar detection.

Mass spectrometry analysis

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF-MS) was used to detect the production of lipopeptides on whole cells and in liquid cultures; i.e. in the cell-free supernatant and in the biosurfactant crude extract. For whole cell analyses, an isolated colony of bacteria grown on TSA solid medium was suspended in 10 µL of matrix solution (10 mg mL^{-1} of α-cyano-4-hydroxycinnamic acid in 70% water, 30% acetonitrile, and 0.1% TFA). For liquid culture analyses, 1 µL of supernatant or crude extract and 1µL of matrix solution were mixed. Then, 1 µL of the prepared samples were spotted in a MALDI-ToF MTP 384 target plate (BrukerDaltonik GmbH, Leipzig, Germany) following the dried-droplet preparation method. Molecular mass analyses were carried out with an Ultraflex MALDI-ToF/ToF mass spectrometer (Bruker, Bremen, Germany) equipped with a smartbeam laser. The masses were measured in reflector positive mode in *m/z* range between 800 and 2000 Da. Analyses were conducted with an accelerating voltage of 25 kV and a deflection mode of matrix suppression at *m/z* 750 Da. The laser power was adjusted to around 30% to above the threshold of ionization.

Effect of pH, temperature and salinity on biosurfactant stability

Biosurfactant stability was studied by testing the liquid surface tension reducing activity and the emulsifying activity of the biosurfactant crude extract solution (0.1% w/v). To test their thermal stability, the biosurfactant solution was set at constant temperatures ranging from 4 to 100 °C for 24 h, and then cooled at room temperature. The pH effect was tested by adjusting the pH of the crude biosurfactant solution

into a range of 2–12 using HCL or NaOH solutions and different buffers: 50 mmol L^{-1}; citrate buffer (pH 3.0–6.0), 50 mmol L^{-1} phosphate buffer (pH 6.0–8.0), and 50 mmol L^{-1} glycine–NaOH buffer (pH 9.0–12.0). The effect of salinity on the biosurfactants stability was investigated by adjusting specific concentrations of NaCl (2–10%, w/v) of crude biosurfactant solution. The surface tension and E24 values for each treatment were performed as described above.

Diesel biodegradation assays

Plastic buckets (7 cm diameter, 15 cm height) were prepared as bioreactor systems for diesel biodegrading assays for each biosurfactant producing strains. Each bioreactor contains 200 mL of sand and is spoiled with 10% (v/v) of diesel oil sterilized by filtration through a 0.2 µm membrane and inoculated with 20 mL of an overnight bacterial culture and then thoroughly mixed together to achieve complete artificial contamination. A bioreactor inoculated with *Rhodococcus erythropolis* T902.1 as a reference strain (Weekers et al. 1999) was used as positive control and a bioreactor fed with free bacteria culture medium as negative control. Bioreactors were continuously flushed by 0.3 bar air and drawn towards a perforate pipe atop the systems. Buckets were daily percolated with M9 minimal medium lacked of carbon sources and having as basis the following composition: Na_2HPO_4 6 g L^{-1}, KH_2PO_4 3 g L^{-1}, NaCl0.5 g L^{-1}, NH_4Cl 1 g L^{-1} and $MgSO_4$ 0.24 g L^{-1}. All bioreactors were incubated at 30 °C for 28 days. Bioreactor contents were aerated by sand turnover every 48 h. Diesel degradation was followed every week by collecting 1 g of contaminated s and mixing it with 2 mL of toluene solvent to measure its optical density by spectrophotometer at 420 nm according to Rahman et al.(2002). Removal percentage of total petroleum hydrocarbon (%TPH) was determined as follows:

$$\%TPH = \left(\frac{ODi - ODx}{ODi}\right) \times 100$$

These experiments were carried out in triplicate.

Chemical oxygen demand (COD) measurement

Diesel mineralization in the sand bioreactor was measured after 28 h of incubation by the test of chemical oxygen demand (COD), using total oxidation of organic carbon with sulphuric acid and potassium dichromate. Around 30 mg of spoiled sand was introduced in COD digestion vials (Hach, USA). Complete oxidation occurred for 2 h at 150 °C. After cooling, absorbance was measured at 565 nm and compared to standards of potassium hydrogenophtalate. Then, sand was washed with distilled water and dried at 105 °C for 24 h and weighted. COD results were expressed in g of oxygen per g of sand. COD removal was calculated as follows:

$$COD\ removal = \left(\frac{COD\ day\,0 - COD\ day\,28}{COD\ day\,0}\right) \times 100$$

Results and discussion

Isolation and screening of biosurfactant producing *Pseudomonas*

Totally eleven *Pseudomonas* strains were isolated on cetrimide-agar plates from samples of oil contaminated soil collected in an auto-mechanical workshop. Morphological observation of these strains showed gram negative bacilli, motile bacteria. Strains were screened for biosurfactant activity by performing drop collapsing tests, surface tension measurements and oil displacement tests on their cell-free supernatant, and emulsion index tests on their biosurfactant crude extract (1% w/v) as these methods are commonly used to detect biosurfactant production by microorganisms (Youssef et al. 2004). Drop collapse test was first used as a simple and rapid method. Five isolates designated by E33b, E38b, E39, E311 and E313 were positive (Table 1) which indicated their potential to produce biosurfactants. Drop collapse assay results did not always correlate with the liquid surface tension reducing activity (Mohammed et al. 2015). So, the cell-free supernatants of all isolated strains were characterised by tensiometry (Table 1). The five drop-collapse-positive isolates (E33b, E38b, E39, E311 and E313) had the highest liquid surface tension reducing activity, the surface tensions of cell-free supernatant cultures were lowered to less than 35 mN m^{-1} which is a criterion of good surfactant (Mulligan 2005). These values were comparable to those reported for culture supernatants of diverse

Table 1 Assessment of biosurfactant and emulsifying activities of *Pseudomonas* spp. isolates obtained from oil contaminated soil

Isolate	Drop collapse test	Surface tension (mN m^{-1})	Oil displacement (mm)	Emulsion index (E24)
E33a	–	55.9 ± 0.2	nd	nd
E33b	+	30.3 ± 1.2	16.2 ± 1.1	53 ± 0.5%
E36	–	47.4 ± 0.8	nd	nd
E37	–	50.3 ± 0.8	nd	nd
E38a	–	49.1 ± 0.2	nd	nd
E38b	+	30.3 ± 1.6	17.0 ± 1.1	62.7 ± 1.1%
E39	+	29.9 ± 0.7	19.3 ± 0.6	79.0 ± 2%
E310	–	52.9 ± 0.5	nd	nd
E311	+	34.0 ± 0.6	19.0 ± 2.7	78.0 ± 0.2%
E312	–	39.6 ± 0.6	nd	nd
E313	+	30.3 ± 0.6	19.3 ± 0.6	79.0 ± 0.4%

nd not determined

biosurfactant producing Pseudomonads, the lowest surface tensions were observed between 24 and 30 mN m^{-1} (Nielsen et al. 2002; Fechtner et al. 2011; Mohammed et al. 2015). Rhamnolipids can reduce the tension surface below 30 mN m^{-1} (Lang and Wullbrandt, 1999; Müller et al. 2012). The lipopopeptides produced by *Pseudomonas,* such as viscosin and arthrofactin, are some of the most efficient biosurfactants by reducing liquid surface tension to 26.5 mN m^{-1} (Neu et al. 1990) and 24 mN m^{-1} (Morikawa et al. 1993) respectively. For comparison, the best known lipopeptide, surfactin produced by *Bacillus subtilis*, reduced liquid surface tension to 27 mN m^{-1} (Arima et al. 1968). The surface tension of the 5 strains free cell supernatants remains stable up to the 1/4th dilution (Fig. 1). This indicated that the concentrations of biosurfactants in all the supernatants were above their F_{CMC} value (Fig. 1). Afterwards, oil displacement and emulsification tests were carried out with the five most surface-active strains. Isolates E39, E311 and E313 performed best in all assays. Their supernatant caused a higher oil displacement and had the highest emulsion index with *n*-hexadecane, i.e. 79 ± 2% for E39 and E313, and 78 ± 2% for E311. These values are high compared to the emulsion index with *n*-hexadecane found for *Pseudomonas* strains, producing rhamnolipids: 70–80% (Aparna et al. 2012; Saikia et al. 2012; Pacwa-Plociniczak et al. 2014), and for a *Pseudomonas* strain, producing lipopeptides: ∼ 58% (de

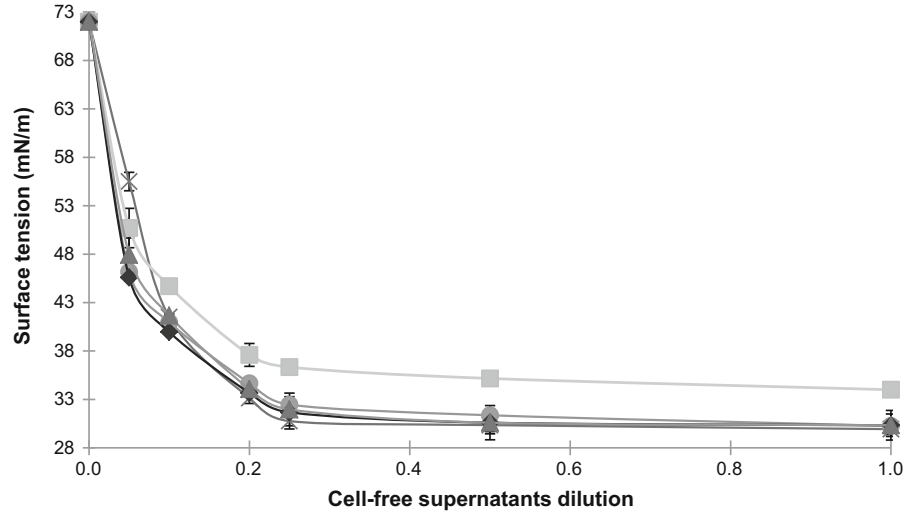


Fig. 1 Plot of surface tension versus serial cell-free supernatant dilutions of *Pseudomonas* isolates E33b (filled triangle), E38b (filled diamond), E39 (times symbol), E311 (filled square) and E313 (filled circle) solutions and F_{CMC} determination

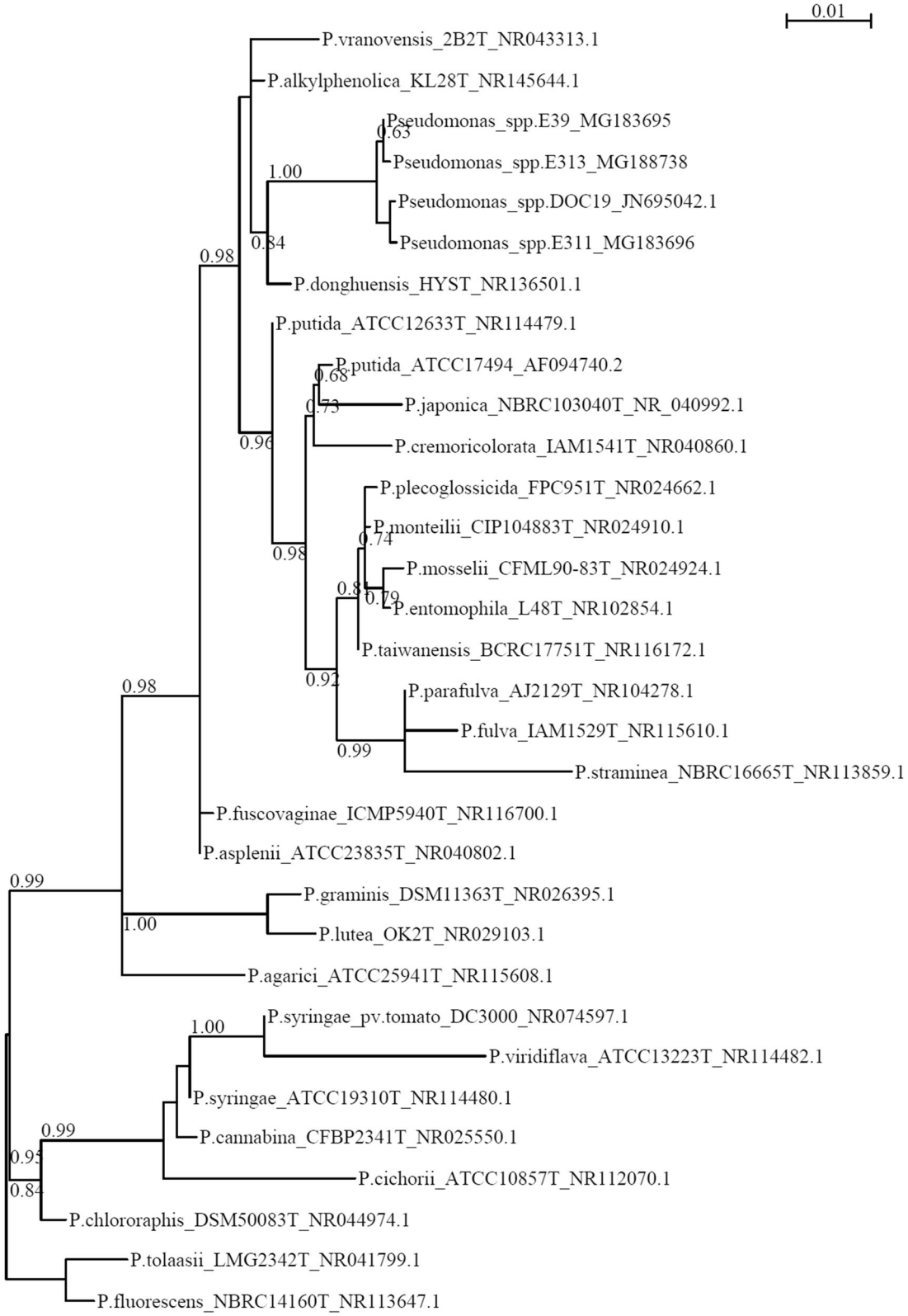
Sousa and Bhosle 2012). Moreover, *Pseudomonas* purified lipopeptides such as viscosin, massetolide A, putisolvin, amphisin (Bak et al. 2015) and pseudofactins (Janek et al. 2010) were shown to be good emulsifiers of *n*-hexadecane. The emulsions resulting from E39, E311 and E313 biosurfactant addition were stable for more than 1 month; this emulsion stability suggests the application of these molecules as good emulsifier agents in industrial domains. For further characterisation, we selected the isolates E39, E311 and E313 that displayed the best emulsion index.

Identification of biosurfactant producing strains

Biosurfactant producing bacteria were identified using biochemical characterisation tests and molecular identification based on whole 16S rRNA gene sequence BLAST analysis. All the three selected strains showed positive reactions to oxidase and arginine dihydrolase. According to the API 20 NE system, E39 was identified as *Pseudomonas* spp., E311 and E313 as *P. putida*. The phylogenetic relationships of E39, E311 and E313 strains with closely related type strains of the genus *Pseudomonas* are shown on the 16S rRNA gene tree (Fig. 2). The 16SrRNA sequence of these three strains were included in the *P. putida* group as defined by Gomila et al. (2015). The most closely related strains were *Pseudomonas* sp. DOC19 (99.8% identity), *P. donghuensis* HYST, *P. alkylphenolica* KL28T and *P. vranovensis* 2B2T (98.7% identity) which are members of the *P. putida* group (Merino et al. 2013; Gao et al. 2015; Mulet et al. 2015; Tvrzova et al. 2006). The position of the strains E39, E311 and E313 in a separate branch of the phylogenetic tree with *Pseudomonas* sp. DOC 19 suggests that all four strains belong to a new species of the *Pseudomonas putida* group which is frequently represented in soils and in water.

Biosurfactant characterisation

To gain further perspicacity about the nature of the produced biosurfactant, TLC analysis was performed. For the crude extracts of isolates E39, E311 and E313, TLC revealed red spots showing a positive reaction when ninhydrin was used as visualization agent, which implies the presence of amino acid groups. No reaction occurred with anthrone reagent, which discards the hypothesis that the selected isolates produced glycolipids. They were then examined for their ability to produce lipopeptide biosurfactants by using MALDI-ToF mass spectrometry which is an efficient device for their detection (Vater et al. 2002). Analyses were performed on whole cells grown on TSA solid medium (Fig. 3), on the supernatant of culture in TSB liquid medium and on crude extract (data not shown). In both solid and liquid media, mass spectra of *Pseudomonas* spp. E39, E311 and E313 revealed series of prominent peaks ranging between *m/z* 1090.7 and 1142.7. All three isolates produce molecules with *m/z* corresponding to syringafactin A (*m/z* 1104.7 $[M+Na]^+$, 1120.7 $[M+K]^+$, 1126.7 $[(M-H) + 2Na]^+$, 1142,7 $[(M-H) + Na + K]^+$). Syringafactins are octalipopeptides linked to C10 or C12 fatty acid, Berti et al. (2007) first identified syringafactins A, B, C, D, E and F which were detected in the mass range from *m/z* 1104.7 to 1146.7 for sodium adducts and from *m/z* 1120.7 to 1162.7 for potassium adducts. Other members of the syringafactin family, named cichofactins, were identified by Pauwelyn et al. (2013); the exact *m/z* of $[M+Na]^+$ of these lipopeptides, are 1131.7 and 1159.7. In this work, mass spectra of three isolates also showed mass peaks at *m/z* 1090.7 $[M+Na]^+$, 1106.7 $[M+K]^+$, 1112.7 $[(M-H) + 2Na]^+$, 1128,7 $[(M-H) + Na + K]^+$, separated by 14 Da from mass peaks at *m/z* 1104.7 $[M+Na]^+$, 1120.7 $[M+K]^+$, 1126.7 $[(M-H) + 2Na]^+$, 1142,7 $[(M-H) + Na + K]^+$ respectively, suggesting that the corresponding molecules varied from each other by the presence of different amino acid residue in the peptide moiety. The intensities of peaks from *m/z* 1090.7 to 1142.7 were lower in the mass profile of strain E311, suggesting it produced lower quantity of syringafactins than strains E39 and E313. The mass profile of strain E39 contained unknown masses from *m/z* 1162.5 to 1189.5 which were not present in other strains. Two of these masses probably correspond to the $[M+Na]^+$ form (*m/z* 1173.5) and to the $[M+K]^+$) form (*m/z* 1189.5) of the same molecule. These differences in mass spectral profiles confirmed that *Pseudomonas* spp. E39, E311 and E313 strains were different although they produced the same syringafactin like molecules. It is interesting to notice that bacteria of the *Pseudomonas putida* group isolated in this work produced biosurfactants of the syringafactin family whereas only a strain of *Pseudomonas syringae*


0.01
P.vranovensis_2B2T_NR043313.1
P.alkylphenolica_KL28T_NR145644.1
Pseudomonas_spp.E39_MG183695
0.63
Pseudomonas_spp.E313_MG188738
1.00
Pseudomonas_spp.DOC19_JN695042.1
0.84
Pseudomonas_spp.E311_MG183696
0.98
P.donghuensis_HYST_NR136501.1
P.putida_ATCC12633T_NR114479.1
P.putida_ATCC17494_AF094740.2
0.68
0.73
P.japonica_NBRC103040T_NR_040992.1
0.96
P.cremoricolorata_IAM1541T_NR040860.1
P.plecoglossicida_FPC951T_NR024662.1
P.monteilii_CIP104883T_NR024910.1
0.74
0.98
P.mosselii_CFML90-83T_NR024924.1
0.8
0.79
P.entomophila_L48T_NR102854.1
P.taiwanensis_BCRC17751T_NR116172.1
0.92
0.98
P.parafulva_AJ2129T_NR104278.1
P.fulva_IAM1529T_NR115610.1
0.99
P.straminea_NBRC16665T_NR113859.1
P.fuscovaginae_ICMP5940T_NR116700.1
P.asplenii_ATCC23835T_NR040802.1
0.99
P.graminis_DSM11363T_NR026395.1
1.00
P.lutea_OK2T_NR029103.1
P.agarici_ATCC25941T_NR115608.1
P.syringae_pv.tomato_DC3000_NR074597.1
1.00
P.viridiflava_ATCC13223T_NR114482.1
P.syringae_ATCC19310T_NR114480.1
0.99
P.cannabina_CFBP2341T_NR025550.1
0.95
P.cichorii_ATCC10857T_NR112070.1
0.84
P.chlororaphis_DSM50083T_NR044974.1
P.tolaasii_LMG2342T_NR041799.1
P.fluorescens_NBRC14160T_NR113647.1

◀**Fig. 2** Maximum-likelihood phylogenetic tree based on partial 16S rRNA gene sequence analysis showing the relationship of biosurfactant producing isolates with phylogenetically close members of the genus *Pseudomonas*. The tree was rooted with *P. tolaassii* LMG 2342T and *P. fluorescens* NRBC 14160T. Branch supports were assessed with the Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT). Only values above 0.6 were displayed

(DC3000) (Berti et al. 2007) and a strain of *Pseudomonas cichorii* (SF1-54) (Pauwelyn et al. 2013) were reported to produce syringafactins. Syringafactin was shown to have biosurfactant properties and to be involved in the swarming properties of the producing strains (Berti et al. 2007; Pauwelyn et al. 2013). However, this lipopeptide was not studied for its bioemulsifying properties and its role in bioremediation. Biosurfactants known to be produced by *P. putida* include rhamnolipids (Tuleva et al. 2005; Martinez-Toledo et al. 2006; Janek et al. 2013), phospholipids (Janek et al. 2013) and the lipopeptides putisolvins (Kuiper et al. 2004; Kruijt et al. 2009) and white-line-inducing principle (WLIP), a member of the viscosin group (Rokni-Zadeh et al. 2012).

Effect of pH, temperature and salinity on biosurfactant stability

The stability of biosurfactants in different environment conditions such as pH, temperature and salinity is an important factor to study, since it affects directly their applicability. The biosurfactants produced by

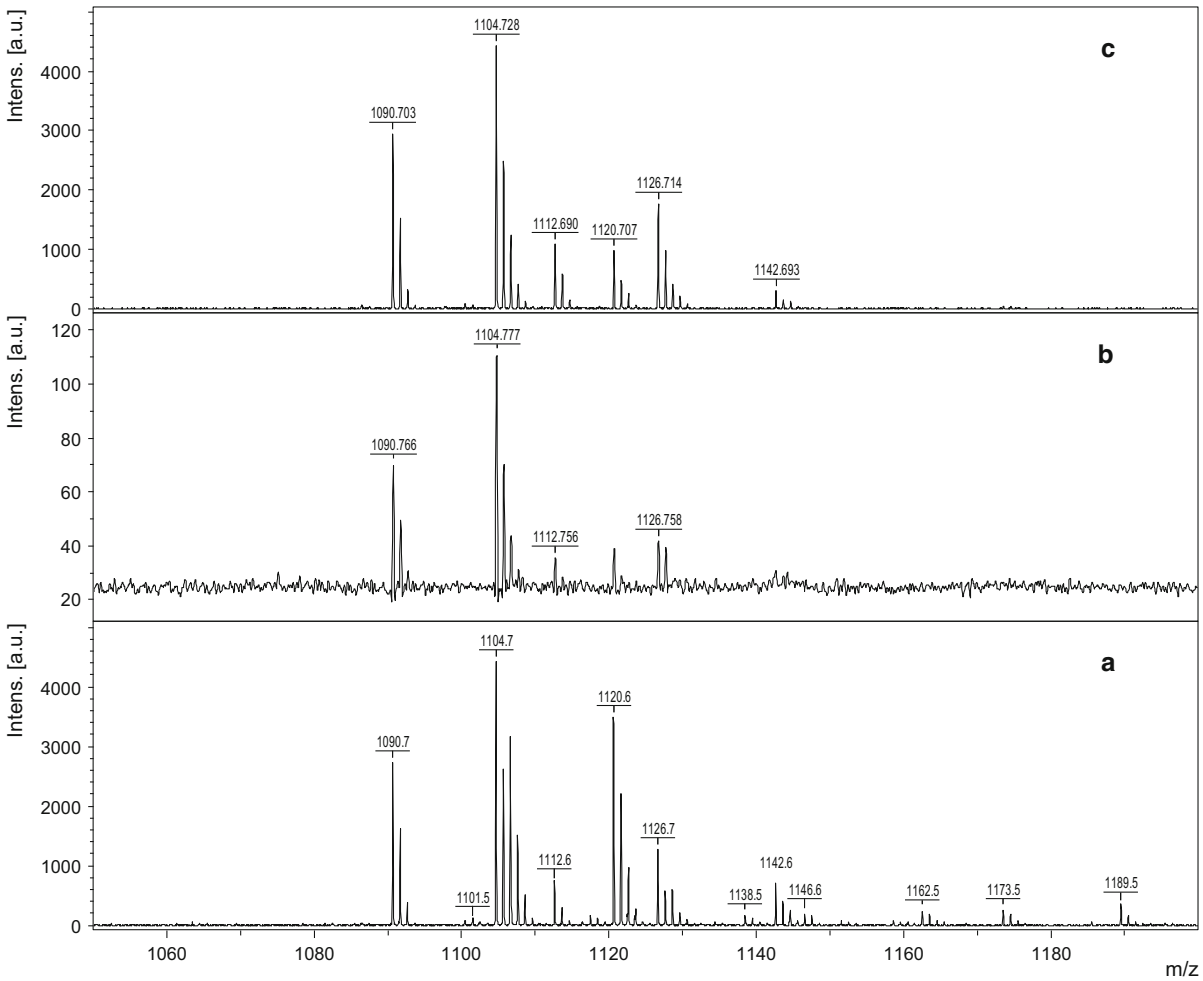


Fig. 3 Whole-cells MALDI-ToF mass spectra of the three selected biosurfactant isolates, **a** *Pseudomonas* spp. E39, **b** *Pseudomonas* spp. E311. **c** *Pseudomonas* spp. E313

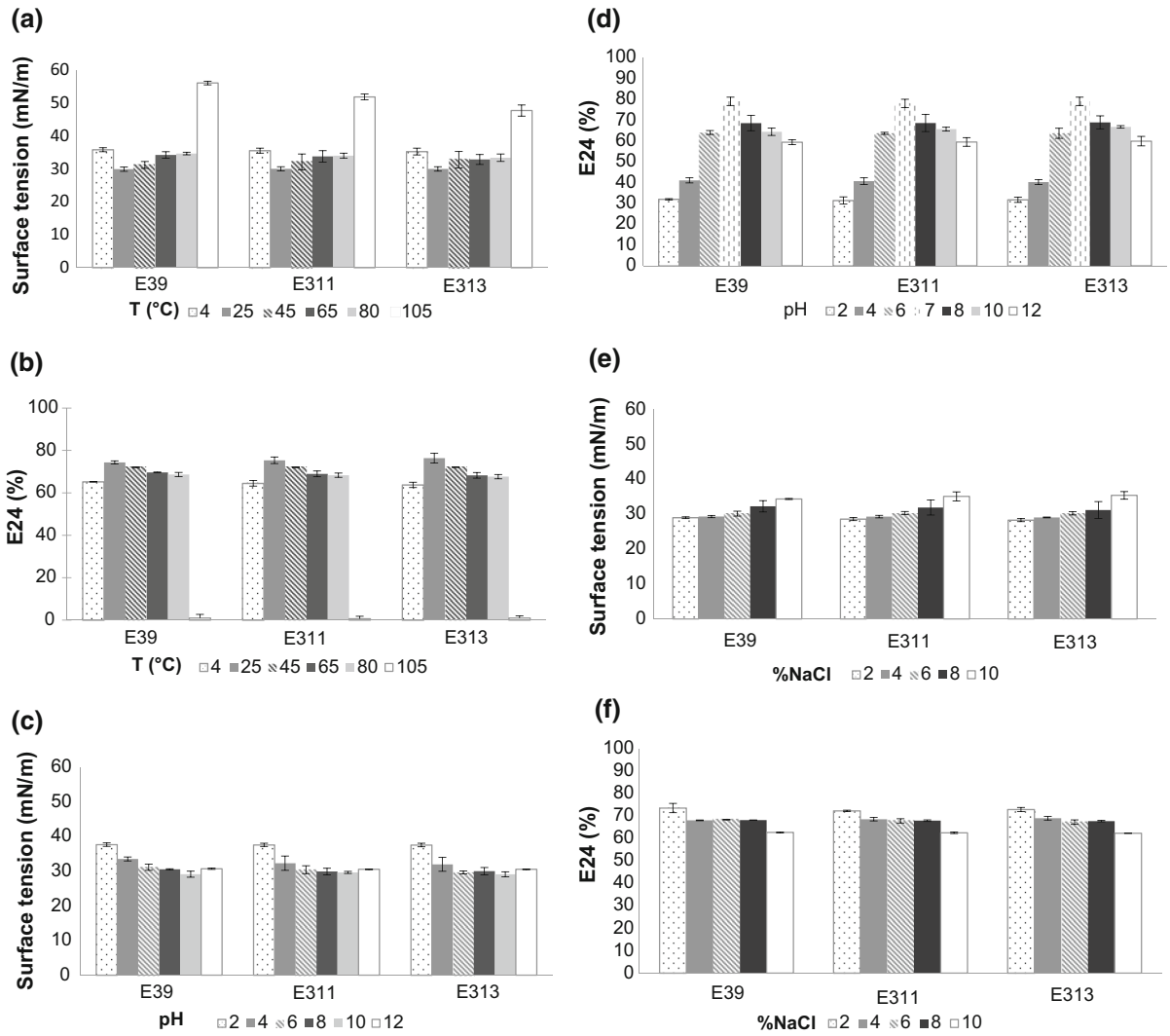


Fig. 4 Study of environmental conditions on biosurfactant crude extracts (0.1% w/v) stability: temperature effect on tensioactive residual activity (**a**) and emulsifying residual activity (**b**), pH effect on tensioactive residual activity (**c**) and emulsifying residual activity (**d**), salinity effect on tensioactive residual activity (**e**) and emulsifying residual activity (**f**) of biosurfactants produced by E39, E311 and E313 strain

Pseudomonas spp. E39, E311 and E313 were shown to be thermostable as they retained their activity after a 24 h treatment at a temperature range of 4–80 °C (Fig. 4a, b). At 105 °C, the tension surface reducing activity decreased. The thermal resistance of biosurfactants is usually high. Rhamnolipids retain good surface tension reducing and emulsifying activities up to 121 °C (Abdel-Mawgoud et al. 2009; Aparna et al. 2012; Saikia et al. 2012) and lipopeptidic biosurfactants up to 80 °C or 100 °C as those produced by *P. nitroreducens* TSB.MJ10 (de Sousa and Bhosle 2012), *Serratia marcescens* NSK-1 (Anyanwu et al. 2011) and *Bacillus subtilis* DM-03 and DM-04 (Mukherjee 2007). The pH stability assays showed that the surface tension reducing and emulsifying activity of the tested biosurfactants remained stable over a pH range of 6–12 (Fig. 4c, d) but was affected by acidic conditions below pH 4. The alkaline pH stability of lipopeptidic biosurfactants has been showed by Anyanwu et al. (2011), de Sousa and Bhosle (2012) and Mukherjee (2007). A decrease of emulsifying activity at acid pH was also observed by de Sousa and Bhosle (2012) and

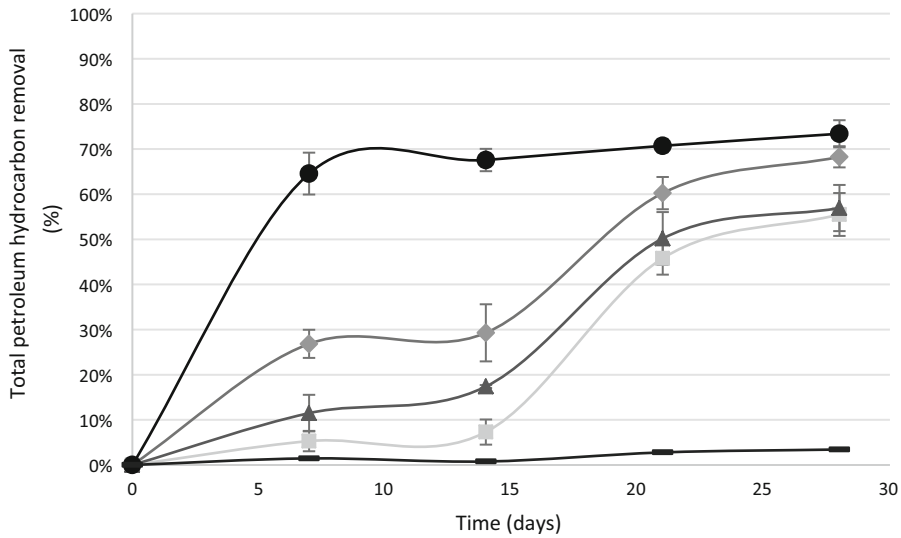


Fig. 5 Study of total petroleum hydrocarbon (TPH) biodegradation by E39 (filled square), E311 (filled diamond) and E313 (filled triangle) strains in diesel contaminated sand comparing with *Rhodococcus erythropolis T902.1* used as positive control (filled circle) and a non-inoculated bucket as a negative control (dashed line)

by Khopade et al. (2012) for an undetermined bioemulsifier. It should also be noticed that the biosurfactant solution became turbid under pH 4 as observed by Khopade et al. (2012).The precipitation of biosurfactant could explain the partial loss of the activity. Rhamnolipids show a higher stability to pH in a range of 2–12 (Abdel-Mawgoud et al. 2009; Aparna et al. 2012; Saikia et al. 2012). The addition of NaCl up to 10% only slightly decreased the activity of biosurfactants (Fig. 4e, f). The tolerance of lipopeptidic or rhamnolipidic biosurfactants has been reported up to 20% or 25% NaCl (Anyanwu et al. 2011; de Sousa and Bhosle 2012; Aparna et al. 2012) The stability of the crude biosurfactants in a wide range of temperature, pH and salinity conditions confirmed their potential to be used in many fields and in particular in bioremediation application.

Diesel biodegradation

In this experiment, we were interested in finding out if the biosurfactant producing isolates might be able to degrade diesel oil since growth on crude oil is often associated with the production of surfactants. Sand bioreactors were contaminated by diesel oil and inoculated with isolates E39, E311, E313 and *Rhodococcus erythropolis* T902.1 as positive control. Biodegradation was monitored by both measuring removal percentage of total petroleum hydrocarbons (% TPH) and COD. In this experiment, strains could only use diesel oil as sole carbon source. At 28 days of biodegradation test, a removal of around 70% of TPH was achieved in bioreactors inoculated with E311 and *R. erythropolis* T902.1. However, *R. erythropolis* T902.1 reached this level of biodegradation sooner (14 days). The removal percentage was lower with E39 and E313 (around 55% of TPH) (Fig. 5). According to mass analyses, strain E311 was the less lipopeptide productive strain whereas its potential for degradation of hydrocarbons was the most effective. There are several hypotheses to be mentioned: (i) the production of biosurfactants is different in the presence of hydrocarbons, (ii) a low production of biosurfactant is sufficient to be effective, (iii) too high production of surfactants could have an inhibitory effect on growth (high hydrocarbons solubility could be toxic for the cell). The removal of COD showed that all strains oxidized diesel in the sand bioreactors (Table 2). The removal of COD of E313 was very close to the removal of TPH which indicates that the

Table 2 Evaluation of diesel degradation by TPH removal and COD removal measurements

Strain	TPH removal (%)	COD removal (%)
E39	55 ± 3	37 ± 10
E311	68 ± 14	22 ± 3
E313	57 ± 19	55 ± 5
R. erythropolis T902.1	75 ± 9	49 ± 8

TPH total petroleum hydrocarbons, *COD* chemical oxygen demand

strain has an efficient diesel mineralization enzymatic potential. The removal of COD was lower than the removal of TPH for the other strains which indicates that diesel is only partially oxidized. These results are comparable with those obtained in other studies (Das and Mukherjee 2007; Mnif et al. 2017) which suggest that these *Pseudomonas* spp. strains could be useful in bioremediation application. The biosurfactants that they produce could enhance oil biodegradation in soils (Pacwa-Płociniczak et al. 2011).

Conclusion

This study enabled to select three *Pseudomonas* spp. isolates from oil contaminated soils for their high emulsifying activities. These isolates (E39, E311 and E313) were closely related to members of the *P. putida* group and their produced biosurfactants were identified as lipopeptides belonging to the syringafactin family. Furthermore, the study showed the high stability of biosurfactants over extreme pH, temperature and salinity conditions and the capacity of syringafactin producing strains to degrade diesel in an artificially contaminated sand. These results suggest the potential of syringafactin for application in bioremediation processes.

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