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The petroleum-degrading bacteria *Alcaligenes aquatilis* strain YGD 2906 as a potential source of lipopeptide biosurfactant

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ABSTRACT

Soummam river sediments were used to isolate a biosurfactant-producing and petroleum-degrading bacterium. The strain was identified as *Alcaligenes aquatilis* YGD 2906 using phenotypic characterization and 16S ribosomal RNA sequencing. The culture supernatant of the isolated strain showed no haemolytic activity had an oiled displacement of 23.66 ± 0.57 mm and Emulsification index (E_{24}) of $68.5\% \pm 0.5\%$. The biosurfactant produced in the minimal medium was extracted by acid precipitation and quantified gravimetrically, with a yield of 4.2 ± 0.01 g/L. The crude Biosurfactant was determined by TLC and MALDI-TOF-MS as a lipopeptide with protein and lipid content of $8.49 \pm 0.19\%$ and $52.66 \pm 1.16\%$ respectively. This lipopeptide structure was confirmed by HPLC-MS/MS. This technique gave two main peak ranges which are typical of surfactins, iturins and fengycin. Tandem mass spectrometry was further used to elucidate the structure of the lipopeptide produced by the strain. The non-haemolytic nature of the lipopeptide produced by this strain offers opportunities for biomedical applications. Further work is needed to optimize production and analyze potential biomedical uses of this lipopeptide including antimicrobial properties.

1. Introduction

Microorganisms capable of degrading hydrophobic substances such as petroleum, are often able to produce natural surfactants or biosurfactants, which are surface-active agents produced by bacteria, yeast and filamentous fungi. These amphiphilic molecules with both hydrophobic and hydrophilic fragments can demonstrate various surface activities, which among other roles, can help to disperse and stabilise hydrophobic substances such as petroleum in water to form an emulsion. The microbial origin and the chemical composition are generally used to classify these biosurfactants which are mainly glycolipids, lipopeptides, lipoproteins, neutral lipids, fatty acids and phospholipids molecules [1].

As biosurfactant are typically capable of emulsifying the hydrophobic (oil or hydrocarbon) phase, it allows their easier dispersion in

water, thus increasing microbial access to these hydrophobic compounds and their availability to biodegradation. They also increase oil mobilization and extraction from the rock formation, a process that can be used for cost-effective solutions to enhance oil recovery technologies [2]. Recently, interest in the novel and natural surfactant has increased, mainly due to their biodegradability and low toxicity [3].

Although the microbial bioremediation of petroleum hydrocarbon is well known [4], these recalcitrant contaminants have limited bioavailability in aqueous systems, thus hindering their utilization or biodegradation by existing microbes [5]. Therefore, the use of biosurfactant producing strains that can degrade petroleum hydrocarbon, does offer the benefit of an accelerated biodegradation process by increasing their bioavailability [5,6]. However, while the benefits offered by biosurfactant producers in petroleum hydrocarbon degradation is well known, studies aimed at characterizing biosurfactants in such processes

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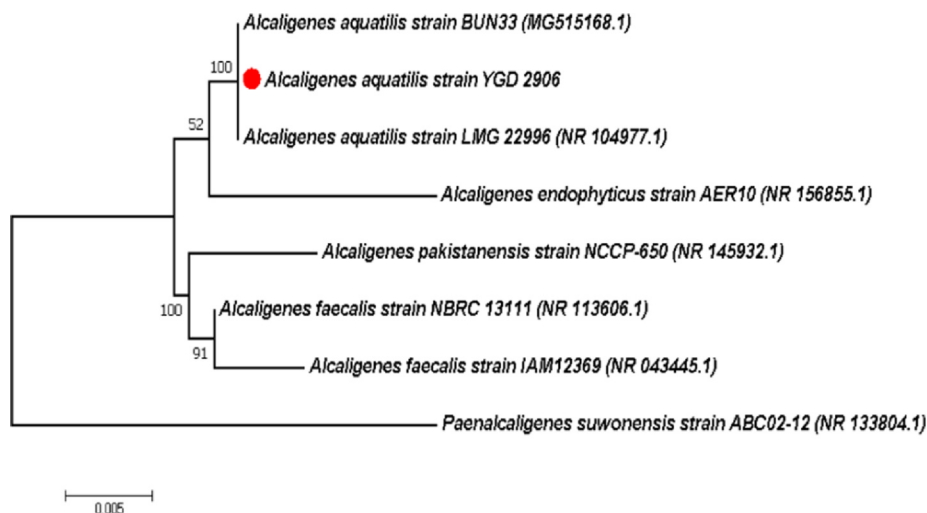


Fig. 1. Neighbour-joining phylogenetic tree showing the taxonomic position of *Alcaligenes aquatilis* strain YGD 2906. *Paenalcaligenes suwonensis* strain ABC02-12 (NR 133804.1) was used as the out-group.

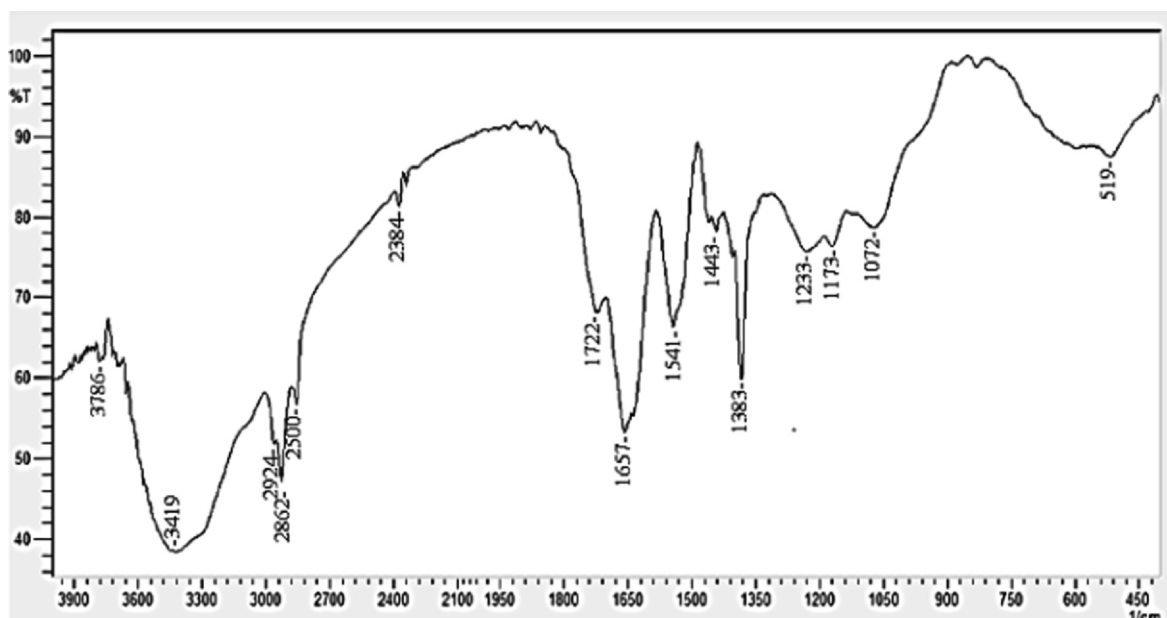


Fig. 2. FT-IR spectrum of the biosurfactant produced by *Alcaligenes aquatilis* strain YGD 2906.

are scarce [7].

Thus, the aims of this study were to isolate petroleum degrading bacteria from Soummam river sediments, investigate their biosurfactant production capacity and fully characterize the biosurfactant produced using different methodologies such as TLC, FTIR, MALDI-TOF and HPLC-MS/MS.

2. Material and methods

2.1. Isolation of petroleum-degrading bacteria

Petroleum-degrading bacteria enrichment and isolation were carried out according to the method reported by [8]. In brief, 10g fresh sediments from Soummam river Skala Bridge in Bejaia, Algeria, which was contaminated with different petroleum derivatives, was added to a 500 ml flat bottle containing 100 ml physiological saline (1% NaCl) and subjected to vigorous agitation for 1–2 h and let stand for 1 h afterwards. Five ml of the supernatant were transferred into Erlenmeyer flasks containing 45 ml Mineral Salt Medium (MSM), containing 3.0 g/L

of Na_2HPO_4 , 1.0 g/L of NH_4NO_3 , 30 g/L of NaCl, 0.7 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g/L of KH_2PO_4 and 1.0 ml of trace element solution. The trace element solution contained: 10 mg/L of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50 mg/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.50 mg/L of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 20 mg/L of CaCl_2 and 30 mg/L of FeCl_3 . This culture medium was supplemented by 2% (v/v) of crude petroleum as an energy and carbon source to enrich petroleum-degrading microbial strains [8]. Afterward, flasks were incubated at 30 °C on a shaker at 150 rpm for 7 days. Five ml of culture medium were transferred to fresh petroleum + MSMmix and incubated at the same conditions to obtain petroleum degrading enriched consortium, this process was carried out for four times. The isolation of pure bacterial strains was carried out by spreading onto petroleum coated MSM agar plates and incubated at 37 °C for 3–5 days. The morphologically distinct colonies were maintained on nutrient agar plates at 4 °C.

2.2. Biosurfactant production test

The detection of biosurfactant production by bacterial isolate was

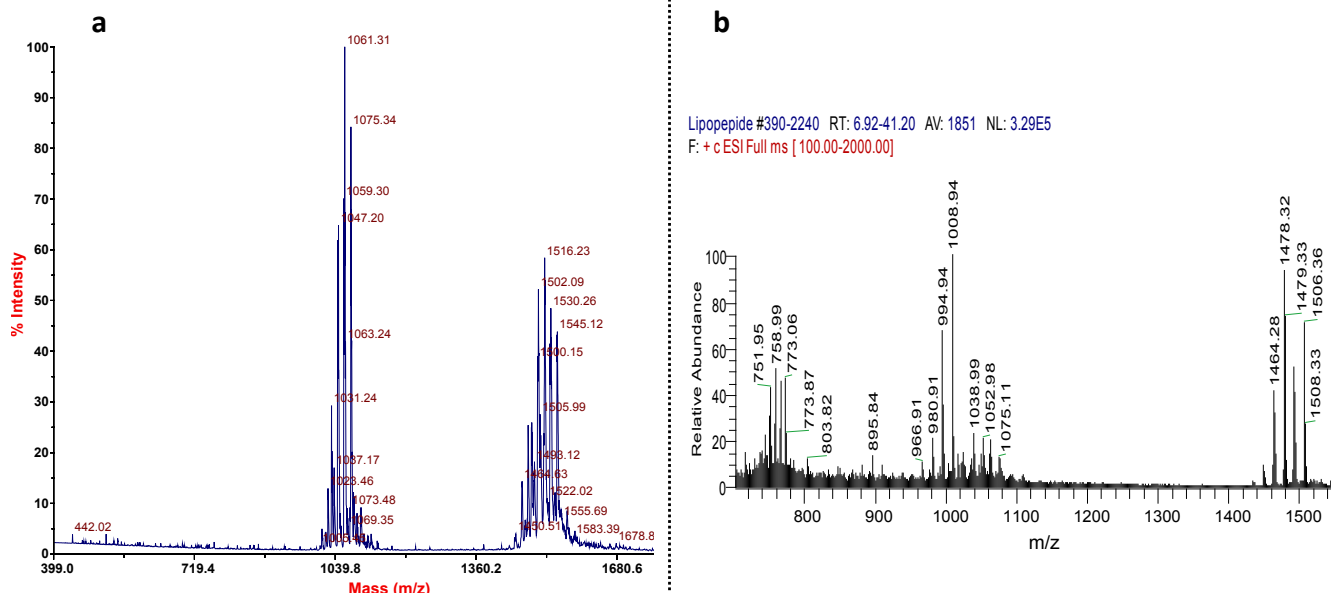


Fig. 3. The a) MALDI-TOF/MS and b) ESI-full MS spectra of extracted lipopeptide, showing two different peak ranges at 1008 to 1105 and 1450 to 1585. ESI-full MS was taken after chromatographic separation as explained in materials and methods and is representative of the entire chromatogram.

Table 1

Lipopeptide assignment from MALDI-TOF/MS.

Mass	Lipopeptide assignment	References
1047.20	C13 Surfactin (M + K) ⁺	[44]
1061.31	C14 Surfactin (M + K) ⁺	proposed
1075.34	C15 Surfactin (M + K) ⁺	[44]
1464.45	C16 Fengycin A (M + H) ⁺	Based on MS/MS analysis
1478.73	C17 Fengycin A (M + H) ⁺	Based on MS/MS analysis
1493.03	C18 Fengycin B (M + H) ⁺	Based on MS/MS analysis
1505.98	C19 Fengycin B (M + H) ⁺	Based on MS/MS analysis

Table 2

Assignment of Lipopeptide from HPLC/MS and their individual relative abundance in accordance to their class.

m/z	Retention time	Relative abundance	Lipopeptide assignment
994	36.52	36.53	C12 Surfactin (M + H) ⁺
1008	39.44	63.47	C13 Surfactin (M + H) ⁺
1464	15.05	14.84	C16 fengycin A (M + H) ⁺
1478	16.07	35.53	C17 fengycin A (M + H) ⁺
1492	15.79	20.13	C16 fengycin B (M + H) ⁺
1506	16.99	29.50	C17 fengycin B (M + H) ⁺

carried out using three screening techniques including haemolytic activity in 5% blood agar plate (the ability to hydrolyze blood cells in blood agar) [9], oil displacement test (the ability to form a clear zone) [10,11] and emulsification activity (The ability to emulsify hydrocarbons) [12]. All experiments were carried out in triplicates.

2.3. Phenotypical identification of petroleum-degrading bacterial isolates

Phenotypical identification of petroleum-degrading bacteria was carried out using Gram staining, citrate utilization, oxidase production, catalase test, methyl red, Vogues Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of carbohydrate, uréase test,

indole production test and nitrate reduction [8].

2.4. 16S ribosomal DNA (rDNA) sequencing of bacterial isolates

The genetic identification of the isolated bacteria was carried out by 16S ribosomal DNA (rDNA) sequencing using universal primers 27F (5'-AGAGATTGATCCTGGCTCT G-3') and 1492R (5'-GGTTTCCTTGTTA CGAC AT-3'). After the comparison between the obtained specific sequences and known 16S rDNA sequences, the sequences were further explored using nucleotide BLAST of NCBI database [13].

2.5. Biosurfactant production and extraction

A single colony of the bacterial strains was transferred into a 100 ml Erlenmeyer flask containing 25 ml MSM broth supplemented with 2% (v/v) of glucose and 0.1 g/L of yeast extract, followed by incubation at 30 °C under 150 rpm agitation for 24 h. Afterward, 5 ml of preculture content was used to inoculate 500 ml of production medium Erlenmeyer flask and incubated at 30 °C on a rotary shaker at 150 rpm for 96 h. Samples were then centrifuged (12,000 g at 4 °C for 20 min) to remove the bacterial cells. The 6 N HCl acid was then used to precipitate the cell-free supernatants to pH 2.0–3.0. Subsequently, the precipitate was collected by centrifugation and the biosurfactant solution was freeze-dried [8].

2.6. Characterization of biosurfactants

2.6.1. Thin layer chromatography analysis

A small amount of the biosurfactants extract was dissolved in chloroform, and analyzed by Thin layer chromatography (TLC) using silica gel 60 coated glass sheet (Silica gel 60F₂₅₄, MERCK, Germany). The plates were developed using solvent mixture: chloroform-methanol-water (65/15/4; v/v/v). Spots were revealed by spraying with the solution of ninhydrin for the detection of compounds with free amino groups, after heating at 110 °C for 5 min [14].

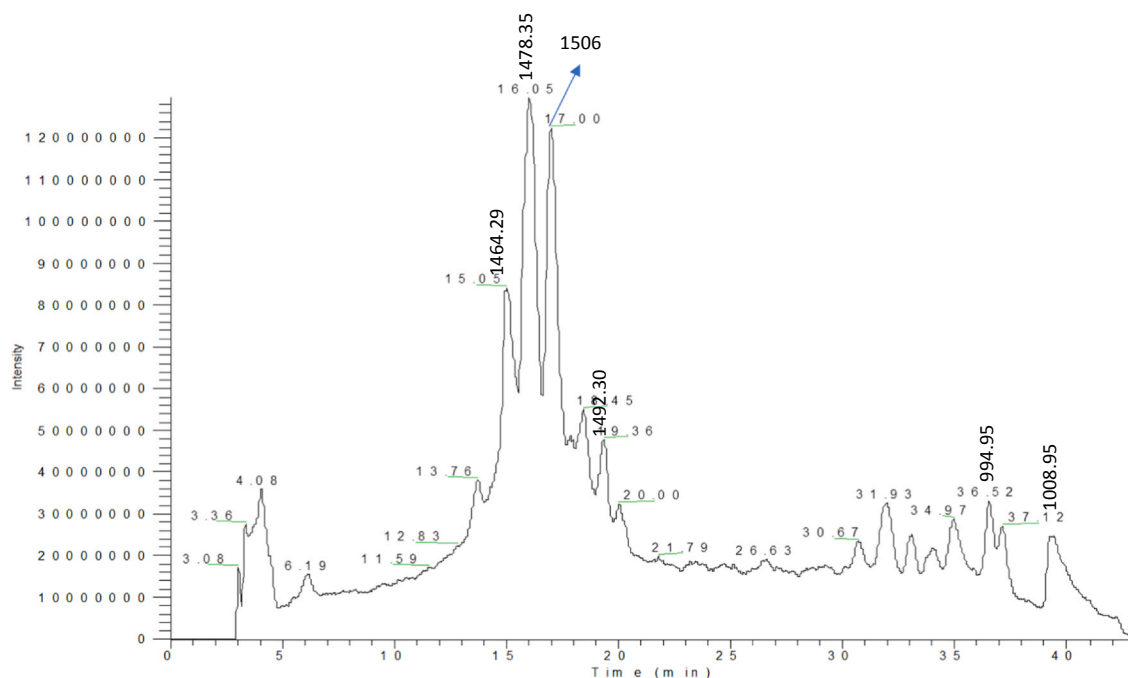


Fig. 4. Chromatogram of Lipopeptide sample, showing the major peaks chosen for tandem mass spectrometric analysis.

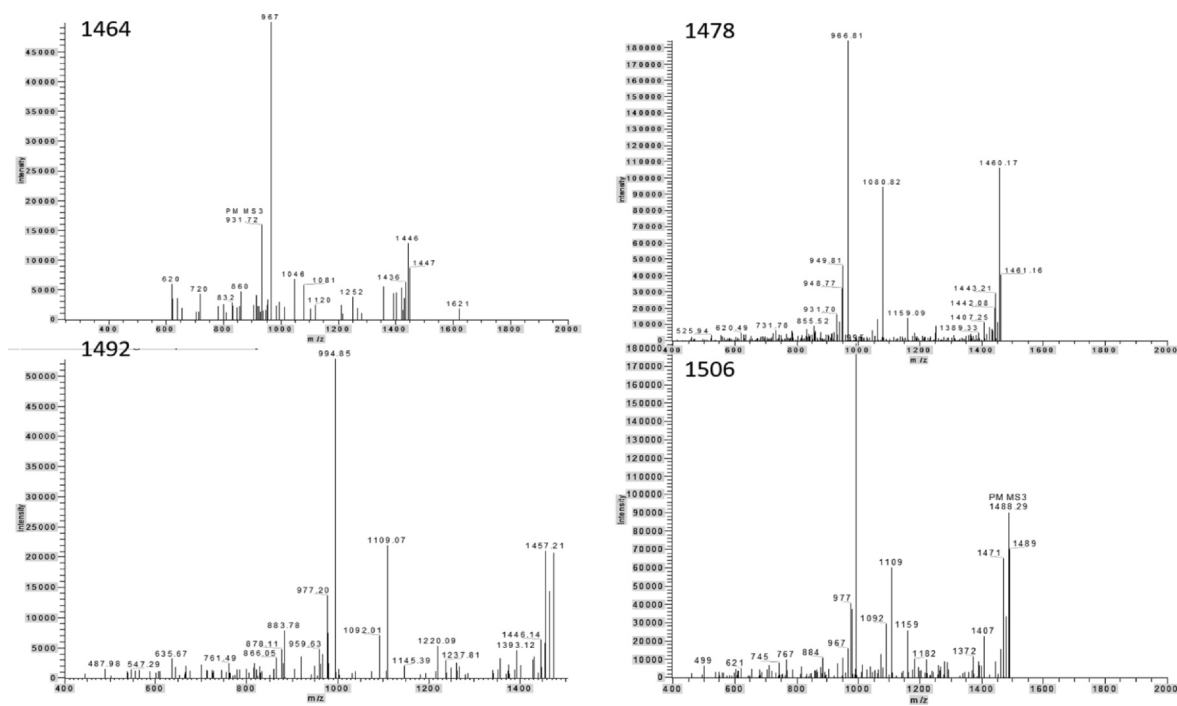


Fig. 5. LC-MS/MS analysis of ions 1464, 1478, 1492 and 1506.

2.6.2. Chemical composition of biosurfactant

The method of Morikawa and co-workers [15] was used to evaluate the lipid content of the biosurfactants. In brief 10 mg of freeze-dried biosurfactant were extracted with mixture of chloroform: methanol: water in the ratios 2: 2: 0.8 (v / v / v). The collected chloroform phase containing the purified lipids was evaporated at 40–60 °C for 20 min. The content of residual lipid was determined gravimetrically.

Total protein content of the biosurfactants was determined according to the Bradford method [16]. Briefly, 1 ml of Bradford reagent was mixed with 20 μ l of the standard gold sample, and the mixture was well shaken. Absorbance was measured at 595 nm and the

concentration of protein was monitored using the calibration curve with Bovine Serum Albumin as standard [17].

2.6.3. Fourier transform infrared spectroscopy characterization

Fourier transform infrared spectroscopy (FTIR) using the potassium bromide (KBr) pellet method was used to identify the functional group in the biosurfactant sample. 0.2 mg biosurfactant samples were mixed with 90 mg of dry KBr to form a very fine powder. This powder was compressed to obtain translucent pellet which could be analyzed by the FTIR system (IRaffinity-1, SHIMADZU). The spectrum was obtained in the range of 400–4000 cm^{-1} . Then, basic functional groups of the

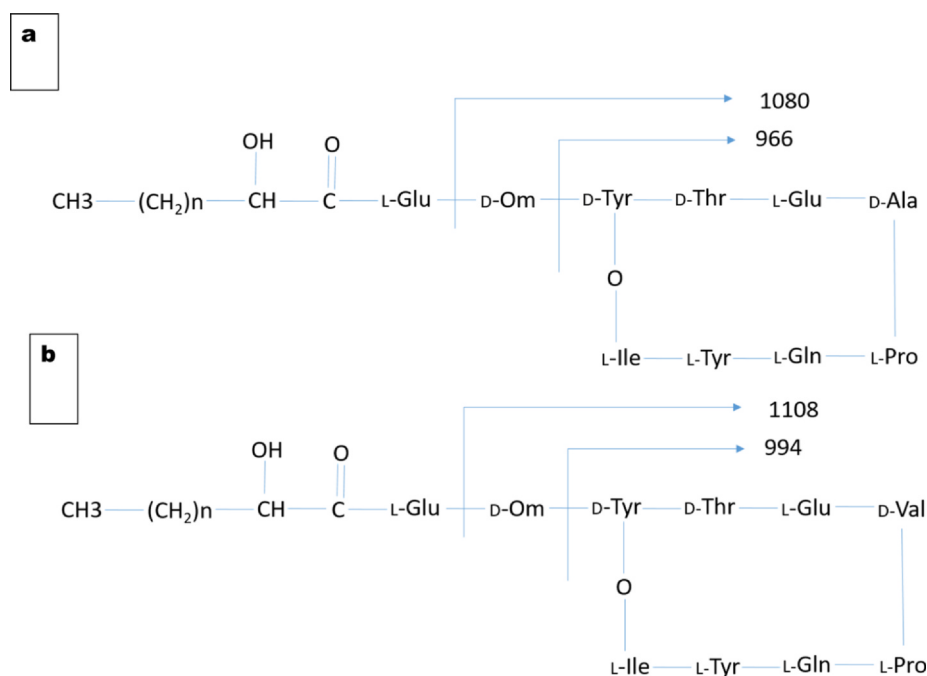


Fig. 6. Structures of Fengycin A ($M + H$)⁺ (a) and Fengycin B ($M + H$)⁺ (b) showing the different daughter ions observed in the MS/MS.

biosurfactant were analyzed according to [17].

2.6.4. Molecular mass determination by MALDI-TOF-MS

Initial analysis was carried out by MALDI-TOF-MS. A Perspective Biosystems Voyager-DE Biospectrometer (Hertfordshire, UK) with a 1 m time-of-flight tube was used for the analysis. The instrument was first calibrated internally using a peptide calibration mix consisting of 4 peptides. A concentration of 1 mg/ml was obtained after reconstitution and dilution of the sample of biosurfactants in methanol. Dihydroxyl benzoic acid was used as the matrix solution [18] and a mass range of 400 Da to 2000 Da was selected, as the most known lipopeptides are within this range [19–21].

2.6.5. HPLC-MS/MS analysis

HPLC-MS method used is as reported previously [22]. The analysis was performed on an LC P400 (ThermoFinnigan) in line with an LCQ quadrupole ion trap mass spectrometer (ThermoFinnigan) equipped with an ESI-source and operated in a positive ionization mode. An Agilent Poroshell 120, EC-C18, 2.1 × 100 mm, 2.7 μm was used as a static phase while the mobile phase consisted of 1% formic acid in water and acetonitrile, respectively. The gradient used is as described by [22].

2.7. Statistical analysis

The experimental data were presented as averages of three replicates, Standard deviations were represented with error bars.

3. Results and discussion

3.1. Screening of biosurfactant production

In this study, we have used the enrichment technique, with mineral salt medium (MSM), to isolate a petroleum-degrading bacteria from the Soummam river sediments in Algeria. As this sediment has been the subject of several hydrocarbon contamination in recent years, microorganisms there have adapted to utilizing petroleum hydrocarbon as their sole carbon and energy sources [8].

Divers tests have been identified for the screening of potential biosurfactant producing isolates. These tests have been extensively

reviewed by various authors and include the following: haemolytic assay, surface/interfacial assay, emulsification assay/index and oil displacement assay [23–25]. Thus, using a number of these screening methodologies, the potential biosurfactant production by the isolated strain was investigated.

First, no haemolysis was observed using the haemolytic activity test of the culture supernatant. While haemolysis is an indication of biosurfactant production, it is not precise and can give a lot of false negative or positive results [23]. Indeed, some biosurfactant has been reported to lack haemolytic activity all together [26]. Das and co-workers [27] showed which showed the production and antimicrobial activity of non-haemolytic lipopeptide species from a marine *Bacillus circulans* strain, noted that non-haemolytic biosurfactants holds huge potential for biomedical applications. The authors further recommended the safe use of purified biotensio-active lipopeptide product as a potential antimicrobial candidate in humans and animals. This result is particularly useful as most potent biosurfactants with antimicrobial properties also possess haemolytic activities, thus disqualifying them as potential drugs for humans and animals [28].

The oil displacement test measures the diameter of the clear zone formed by the surface activity of the surfactant solution tested, with respect to the oil. A high surface activity of the biosurfactant is indicated by a large diameter of the oil displacement. The supernatant of the isolated strain resulted a diameter of 23.66 ± 0.57 mm, which indicates the presence of high concentrations of surface-active compounds. The microbial production of surfactants by microorganisms growing on crude oil and other hydrophobic substances has been frequently reported [29,30]. Furthermore, the emulsification index (E_{24}) of the culture supernatant for the isolated strain was found to be very interesting. The value of the emulsification index (E_{24}) was $68.5 \pm 0.5\%$, this result was higher than the positive control (SDS) ($64.77 \pm 1.6\%$). The crude oil emulsification in water is a prerequisite which opens the way for the biodegradation of this environmental pollutant by many bacteria. It improves the bioavailability of petroleum and thus increases the rate of biodegradation [31–33].

3.2. Identification of the isolate

The petroleum degrading bacterial isolate was identified as

Alcaligenes aquatilis strain YGD2906, according to 16S rDNA gene sequences and Neighbor-joining phylogenetic analysis (Fig. 1). This strain was confirmed as gram-negative, while oxidase, catalase and urease production tests were positive. All other tests such as citrate utilization, methyl red, Vogues Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of carbohydrate, indole production test and nitrate reduction were negative.

The evolutionary history was inferred using the Neighbour-Joining method [34]. The optimal tree with the sum of branch length = 0.06473377 is shown. The percentage of bootstrap value (1000 replicates) is shown next to the branches [35]. The evolutionary distances were computed using the p-distance method [36] and are in the units of the number of base differences per site. Among the eight nucleotide sequences, the positions containing gaps and missing data were excluded from the analysis, resulting in a total of 1371 positions. MEGA7 was used for the evolutionary analyses [37]. The sequence is deposited in Genbank under the accession number MT103125.

3.3. Biosurfactant production, extraction and quantification

Production of biosurfactant by the strain *Alcaligenes aquatilis* YGD2906 was carried out in MSM broth supplemented with 2% (v/v) glucose and 0.1 g/L of yeast extract. Cells were incubated at 30 °C with stirring at 150 rpm for 96 h. After extraction by acid precipitation, a total of 4.2 ± 0.01 g/L crude freeze-dried biosurfactant was obtained. The value of the emulsification index ($E_{24\%}$) of this biosurfactant was $73.23 \pm 1.61\%$, which was higher than the positive control (SDS) ($64.77 \pm 1.6\%$). However, this quantity is quite appreciable compared to that observed by others [38,39].

3.4. Characterization of biosurfactant produced by *Alcaligenes aquatilis* YGD2906

3.4.1. Thin layer chromatography (TLC) analysis and chemical composition of biosurfactant

TLC spots were visible on plates after spraying with ninhydrin reagent, indicating the presence of free amino acids. These results suggest the lipopeptide nature of the bio-surfactant produced by the isolated strain, similar to those reported previously for other bacterial strains [27,40,41]. The lipopeptide nature of the biosurfactant was further confirmed by protein and lipid content determination, which was $8.49 \pm 0.19\%$ and $52.66 \pm 1.16\%$ respectively. These results confirm the lipopeptide nature of the biosurfactant produced by the isolated *Alcaligenes aquatilis* YGD 2906.

3.4.2. Fourier transform infrared spectroscopy (FTIR) characterization

The functional groups present in the produced biosurfactant were identified by FTIR analysis. The FTIR spectrum (Fig. 2), illustrates the presence of both an aliphatic and peptide chain.

The peaks at 3419 cm^{-1} and 1657 cm^{-1} corresponded to stretching and bending of N-H group, respectively. The peaks at 2924 and 2862 cm^{-1} indicated the presence of methyl and methylene, respectively. The peaks at 1722 , 1443 , 1383 , 1233 cm^{-1} showed the stretching of C = O, aliphatic chain ($-\text{CH}_2-$), C-H bend, acyl and phenyl C-O, respectively. A peak at 1072 cm^{-1} pointed out the presence of alkoxy group. FTIR results, therefore, indicated the presence of aliphatic hydrocarbon in combination with peptide group, which is the characteristic feature of lipopeptide biosurfactants such as surfactin [42,43].

3.4.3. Molecular mass determination by MALDI-TOF-MS

The results from the MALDI-TOF-MS are presented in Fig. 3a. Two main peak ranges were identified: 1008 to 1105 and 1450 to 1585. These peaks are typical of Surfactins, Iturins and Fengycins. The major peaks within these range and their potential lipopeptide assignments are shown in Table 1 [44]. The Table shows mass structures 1047, 1061

and 1075 typical for surfactins containing 13, 14 and 15 carbon atoms, respectively. It also shows mass structures 1464, 1478, 1493, and 1505 which we believe represents different Fengycin type surfactants based on their MS/MS analysis which generated molecular structure breakdown expected for these types of molecules as detailed below.

3.4.4. HPLC-MS/MS analysis

Following results from the MALDI-TOF-MS, further characterization was carried out using HPLC-MS to separate the individual species. The full ESI-MS is presented in Fig. 3b and showed 2 distinct peak patterns as those observed in MALDI-TOF/MS with additional peaks at 751 to 773 corresponding to the double charged 1464 to 1506 [45] having a proposed K^+ . The results from HPLC-MS are presented in Table 2. Other peaks were also identified but these have a series of contaminating fractions that could not be resolved and hence are not presented in Table 2.

To further characterize the lipopeptides, another HPLC-MS run was carried out with an added setting to characterise all relevant peaks (Fig. 4) by MS/MS and further characterise relevant MS/MS products by MS3 with dissociation energy of 35. At the collision energy used, only peaks 1464, 1478, 1492 and 1506 gave values that can be further characterized (Fig. 5). These peaks were initially thought to be C16 to C19 Fengycin A ($\text{M} + \text{H}^+$), respectively. However, the collision induced dissociation (CID) showed that 1464 and 1478 has daughter ions at 966 and 1080 while 1492 and 1506 has daughter ions at 994 and 1108. The latter are indicative of C16 and C17 Fengycin A ($\text{M} + \text{H}^+$) with D-Ala at position 6 while the former is indicative of C16 and C17 Fengycin B ($\text{M} + \text{H}^+$) with D-Val at position 6 (Fig. 6a and b) [22].

4. Conclusion

This study was the first research about introducing *Alcaligenes aquatilis* YGD 2906 as a petroleum-degrading bacteria strain, capable of biosurfactant production. Different methods such as TLC, FTIR, MALDI-TOF/MS were used to characterize the biosurfactant produced by this strain. The results showed that this strain can produce several lipopeptide species particularly Surfactins and Fengycins. Following chromatographic separation, MS/MS analysis was used to resolve the chemical structure of Fengycin A and B, produced by the isolated strain. The produced lipopeptide also showed no haemolytic activities, thus making it a desirable candidate for biomedical applications, including use as antimicrobial agents as previously suggested for non-haemolytic lipopeptides by [27].

CRedit authorship contribution statement

Drifa Yalaoui-Guellal: Conceptualization, Methodology, Software. **Samira Fella-Temzi:** Software. **Salima Djafri-Dib:** Software. **Sunil Kumar Sahu:** Software. **Victor U. Irorere:** Software. **Ibrahim M. Banat:** Software. **Khodir Madani:** Software.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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