

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

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22 **Abstract**

23 Soummam river sediments were used to isolate a biosurfactant-producing and petroleum-
24 degrading bacterium. This strain was identified as *Alcaligenes aquatilis* YGD 2906 using
25 phenotypic characterization and 16S ribosomal RNA sequencing. The culture supernatant
26 of the isolated strain showed no haemolytic activity had an oiled displacement of $23.66 \pm$
27 0.57 mm and Emulsification index (E24) of $68.5\% \pm 0.5\%$. The biosurfactant produced
28 in minimal medium was extracted by acid precipitation and quantified gravimetrically,
29 with a yield of 4.2 ± 0.01 g / L. The crude Biosurfactant was determinate by TLC and
30 MALDI-TOF-MS as a lipopeptide with protein and lipid content of $8.49 \pm 0.19\%$ and
31 $52.66 \pm 1.16\%$ respectively. This lipopeptide structure was confirmed by HPLC-MS/MS.
32 This technique gave two main peak ranges which are typical of surfactins, iturins and
33 fengycin. Tandem mass spectrometry was further used to elucidate the structure of the
34 lipopeptide produced by the strain. The non-haemolytic nature of the lipopeptide
35 produced by this strain offers opportunities for biomedical applications. Further work is
36 needed to optimize production and analyze potential biomedical uses of this lipopeptide
37 including antimicrobial properties.

38 **Keywords:** Petroleum biodegradation, *Alcaligenes aquatilis* strain YGD 2906,
39 Biosurfactant production, MALDI-TOF/MS analysis, HPLC-MS/MS.

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44 **1. Introduction**

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

54 As biosurfactant are typically capable of emulsifying the hydrophobic (oil or
55 hydrocarbon) phase, it allows their easier dispersion in water, thus increasing microbial
56 access to these hydrophobic compounds and their availability to biodegradation. They
57 also increase oil mobilization and extraction from rock formation, a process that can be
58 used for cost-effective solutions to enhance oil recovery technologies [2]. Recently,
59 interest in the novel and natural surfactant has increased, mainly due to their
60 biodegradability and low toxicity [3].

61 Although the microbial bioremediation of petroleum hydrocarbon is well known [4],
62 these recalcitrant contaminants have limited bioavailability in aqueous systems, thus
63 hindering their utilisation or biodegradation by existing microbes [5]. Therefore, the use
64 of biosurfactant producing strains that can degrade petroleum hydrocarbon, does offer the
65 benefit of an accelerated biodegradation process by increasing their bioavailability [5, 6].
66 However, while the benefits offered by biosurfactant producers in petroleum hydrocarbon

67 degradation is well known, studies aimed at characterising biosurfactants in such
68 processes are scarce [7].

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

73 **2. Material and methods**

74 **2.1. Isolation of petroleum-degrading bacteria**

75 Petroleum-degrading bacteria enrichment and isolation were carried out according to the
76 method reported by [8]. In brief, 10g fresh sediments from Soummam river Skala Bridge
77 in Bejaia, Algeria, which was contaminated with different petroleum derivatives, was added
78 to a 500 ml flat bottle containing 100 ml physiological saline (1% NaCl) and subjected to
79 vigorous agitation for 1–2 h and let stand for 1 h afterwards. Five ml of the supernatant
80 were transferred into Erlenmeyer flasks containing 45 ml Mineral Salt Medium
81 (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
82 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g/L of KH_2PO_4 and 1.0 ml of trace element solution. The trace element
83 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
84 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 20 mg/L of CaCl_2 and 30 mg/L of FeCl_3 . This culture medium was
85 supplemented by 2% (v/v) of crude petroleum as an energy and carbon source to enrich
86 petroleum-degrading microbial strains [8]. Afterward, flasks were incubated at 30 °C on
87 a shaker at 150 rpm for 7 days. Five ml of culture medium were transferred to fresh
88 petroleum + MSM mix and incubated at the same conditions to obtain petroleum
89 degrading enriched consortium, this process was carried out for four times. The isolation

90 of pure bacterial strains was carried out by spreading onto petroleum coated MSM agar
91 plates and incubated at 37°C for 3-5 days. The morphologically distinct colonies were
92 maintained on nutrient agar plates at 4°C.

93 **2.2. Biosurfactant production test**

94 The detection of biosurfactant production by bacterial isolate was carried out using three
95 screening techniques including haemolytic activity in 5% blood agar plate (the ability to
96 hydrolyze blood cells in blood agar) [9], oil displacement test (the ability to form a clear
97 zone) [10, 11] and emulsification activity (The ability to emulsify hydrocarbons) [12].
98 All experiments were carried out in triplicates.

99 **2.3. Phenotypical identification of petroleum-degrading bacterial isolates**

100 Phenotypical identification of petroleum-degrading bacteria was carried out using Gram
101 staining, citrate utilization, oxidase production, catalase test, methyl red, Vogues
102 Proskauer test, gelatine liquefaction test, triple sugar iron test, fermentation of
103 carbohydrate, urease test, indole production test and nitrate reduction [8].

104 **2.4. 16S ribosomal DNA (rDNA) sequencing of bacterial isolates**

105 The genetic identification of the isolated bacteria was carried out by 16S ribosomal DNA
106 (rDNA) sequencing using universal primers 27F (5'-AGAGATTGATCCTGGCTCT G-
107 3') and 1492R (5'-GGTTCCTTGTTACGAC AT-3'). After the comparison between the
108 obtained specific sequences and known 16S rDNA sequences, the sequences were further
109 explored using nucleotide BLAST of NCBI database [13].

110 **2.5. Biosurfactant production and extraction**

111 A single colony of the bacterial strains were transferred into a 100 ml Erlenmeyer flask
112 containing 25 ml MSM broth supplemented with 2% (v/v) of glucose and 0.1 g/L of yeast
113 extract, followed by incubation at 30°C under 150 rpm agitation for 24 h. Afterward, 5ml
114 of preculture content was used to inoculate 500ml of production medium Erlenmeyer
115 flask and incubated at 30°C on a rotary shaker at 150 rpm for 96h. Samples were then
116 centrifuged (12,000 g at 4°C for 20 min) to remove the bacterial cells. The 6N HCl acid
117 was then used to precipitate the cell-free supernatants to pH 2.0 - 3.0. Subsequently, the
118 precipitate was collected by centrifugation and the biosurfactant solution was freeze-dried
119 [8].

120 **2.6 Characterization of biosurfactants**

121 **2.6.1. Thin layer chromatography analysis**

122 A small amount of the biosurfactants extract was dissolved in chloroform, and analyzed
123 by Thin layer chromatography (TLC) using silica gel 60 coated glass sheet (Silica gel
124 60F₂₅₄, MERCK, Germany). The plates were developed using solvent mixture:
125 chloroform-methanol-water (65/15/4; v/v/v). Spots were revealed by spraying with
126 solution of ninhydrin for the detection of compounds with free amino groups, after
127 heating at 110 °C for 5 min [14].

128 **2.6.2. Chemical composition of biosurfactant**

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

134 Total protein content of the biosurfactants was determined according to the Bradford
135 method [16]. Briefly, 1 ml of Bradford reagent was mixed with 20 μ l of the standard gold
136 sample, and the mixture was well shaken. Absorbance was measured at 595 nm and the
137 concentration of protein was monitored using the calibration curve with Bovine Serum
138 Albumin as standard [17].

139 **2.6.3. Fourier Transform Infrared Spectroscopy characterization**

140 Fourier transform infrared spectroscopy (FTIR) using the potassium bromide (KBr) pellet
141 method was used to identify the functional group in the biosurfactant sample. 0.2 mg
142 biosurfactant samples were mixed with 90 mg of dry KBr to form a very fine powder.
143 This powder was compressed to obtain translucent pellet which could be analyzed by the
144 FTIR system (IRaffinity-1, SHIMADZU). The spectrum was obtained in the range of
145 400–4000 cm^{-1} . Then, basic functional groups of the biosurfactant were analyzed
146 according to [17].

147 **2.6.4. Molecular mass determination by MALDI-TOF-MS**

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

155 **2.6.5. HPLC-MS/MS analysis**

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

162 **2.7. Statistical analysis**

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

165 **3. Results and Discussion**

166 **3.1. Screening of Biosurfactant production**

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

172 Divers tests have been identified for the screening of potential biosurfactant producing
173 isolates. These tests have been extensively reviewed by various authors and include the
174 following: haemolytic assay, surface/interfacial assay, emulsification assay/index and oil
175 displacement assay [23-25]. Thus, using a number of these screening methodologies, the
176 potential biosurfactant production by the isolated strain was investigated.

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

188 The oil displacement test measures the diameter of the clear zone formed by the surface
189 activity of the surfactant solution tested, with respect to the oil. A high surface activity of
190 the biosurfactant is indicated by a large diameter of the oil displacement. The supernatant
191 of the isolated strain gave a diameter of 23.66 ± 0.57 mm, which indicates the presence
192 of high concentrations of surface-active compounds. The microbial production of
193 surfactants by microorganisms growing on crude oil and other hydrophobic substances
194 has been frequently reported [29, 30]. Furthermore, the emulsification index (E_{24}) of the
195 culture supernatant for the isolated strain was found to be very interesting. The value of
196 the emulsification index (E_{24}) was $68.5 \pm 0.5\%$, this result was higher than the positive
197 control (SDS) ($64.77 \pm 1.6\%$). The crude oil emulsification in water is a prerequisite which
198 opens the way for the biodegradation of this environmental pollutant by many bacteria. It
199 improves the bioavailability of petroleum and thus increases the rate of biodegradation
200 [31-33].

201 **3.2. Identification of the isolate**

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

208 The evolutionary history was inferred using the Neighbour-Joining method [34]. The
209 optimal tree with the sum of branch length = 0.06473377 is shown. The percentage of
210 bootstrap value (1000 replicates) is shown next to the branches [35]. The evolutionary
211 distances were computed using the p-distance method [36] and are in the units of the
212 number of base differences per site. Among the eight nucleotide sequences, the positions
213 containing gaps and missing data were excluded from the analysis, resulting in a total of
214 1371 positions. MEGA7 was used for the evolutionary analyses [37]. The sequence is
215 deposited in Genbank under accession number MT103125.

216 **3.3. Biosurfactant production, extraction and quantification**

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

224 **3.4. Characterization of biosurfactant produced by *Alcaligenes aquatilis* YGD2906**

225 **3.4.1. Thin layer chromatography (TLC) analysis and Chemical composition of** 226 **biosurfactant**

227 TLC spots were visible on plates after spraying with ninhydrin reagent, indicating the
228 presence of free amino acids. These results suggest the lipopeptide nature of the bio-
229 surfactant produced by the isolated strain, similar to those reported previously for other
230 bacterial strains [27, 40, 41]. The lipopeptide nature of the biosurfactant was further
231 confirmed by protein and lipid content determination, which was $8.49 \pm 0.19\%$ and 52.66
232 $\pm 1.16\%$ respectively. These results confirm the lipopeptide nature of the biosurfactant
233 produced by the isolated *Alcaligenes aquatilis* YGD 2906.

234 **3.4.2. Fourier Transform Infrared Spectroscopy (FTIR) Characterization**

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

238 The peaks at 3419 cm^{-1} and 1657 cm^{-1} corresponded to stretching and bending of N-H
239 group, respectively. The peaks at 2924 and 2862 cm^{-1} indicated the presence of methyl
240 and methylene, respectively. The peaks at 1722 , 1443 , 1383 , 1233 cm^{-1} showed the
241 stretching of C=O, aliphatic chain (-CH₂-), C-H bend, acyl and phenyl C-O,
242 respectively. A peak at 1072 cm^{-1} pointed out the presence of alkoxy group. FTIR results,
243 therefore, indicated the presence of aliphatic hydrocarbon in combination with peptide
244 group, which is the characteristic feature of lipopeptide biosurfactants such as surfactin
245 [42, 43].

246 3.4.3. Molecular mass determination by MALDI-TOF-MS

247 The results from the MALDI-TOF-MS are presented in Fig.3a. Two main peak ranges
248 were identified: 1008 to 1105 and 1450 to 1585. These peaks are typical of Surfactins,
249 Iturins and Fengycins. The major peaks within these range and their potential lipopeptide
250 assignments are shown in Table 1 [44]. The Table shows mass structures 1047,1061 and
251 1075 typical for surfactins containing 13,14 and 15 carbon atoms, respectively. It also
252 shows mass structures 1464, 1478,1493, and 1505 which we believe represents different
253 Fengycin type surfactants based on their MS/MS analysis which generated molecular
254 structure breakdown expected for these type of molecules as detailed below.

255 3.4.4. HPLC-MS/MS analysis

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

263 To further characterize the lipopeptides, another HPLC-MS run was carried out with an
264 added setting to characterise all relevant peaks (Fig. 4) by MS/MS and further characterise
265 relevant MS/MS products by MS3 with dissociation energy of 35. At the collision energy
266 used, only peaks 1464, 1478, 1492 and 1506 gave values that can be further characterized
267 (Fig.5). These peaks were initially thought to be C16 to C19 Fengycin A $(M+H)^+$,
268 respectively. However, the collision induced dissociation (CID) showed that 1464 and

269 1478 has daughter ions at 966 and 1080 while 1492 and 1506 has daughter ions at 994
270 and 1108. The latter are indicative of C16 and C17 Fengycin A (M+H)⁺with D-Ala at
271 position 6 while the former is indicative of C16 and C17 Fengycin B (M+H)⁺with D-Val
272 at position 6 (Fig.6a and b) [22].

273 **4. Conclusion**

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

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427 **Table 1**

428 Lipopeptide assignment from MALDI-TOF/MS

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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

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442 **Table 2**443 Assignment of Lipopeptide from LC/MS and their individual relative abundance in
444 accordance to their class

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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) ⁺

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460 **Figure Captions**

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

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

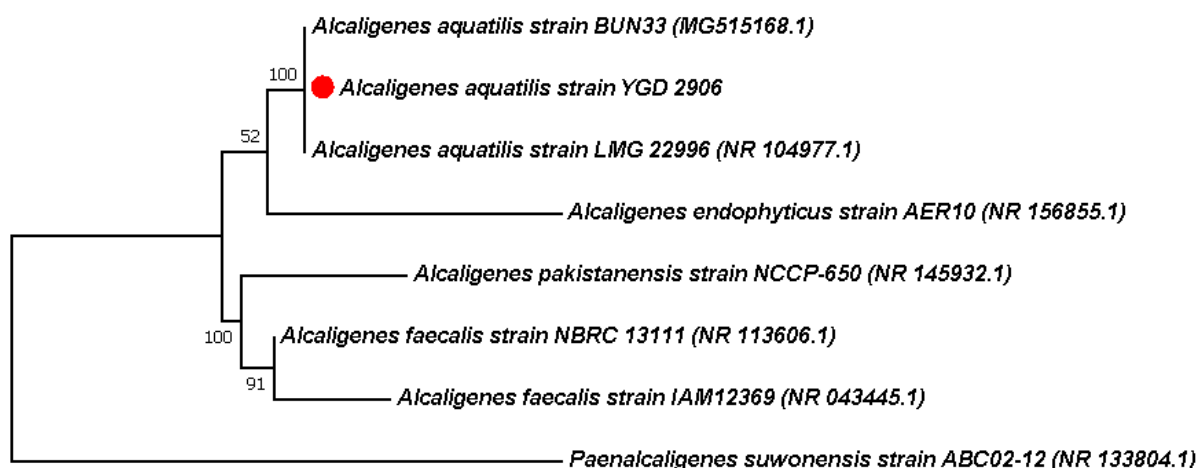
466 **Fig.3.** The a) MALDI-TOF/MS and b) ESI-full MS spectra of extracted lipopeptide,
467 showing two different peak ranges at 1008 to 1105 and 1450 to 1585. ESI-full MS was
468 taken after chromatographic separation as explained in materials and methods and is
469 representative of the entire chromatogram.

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

472 **Fig.5.** HPLC-MS/MS analysis of ions 1464, 1478, 1492 and 1506

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

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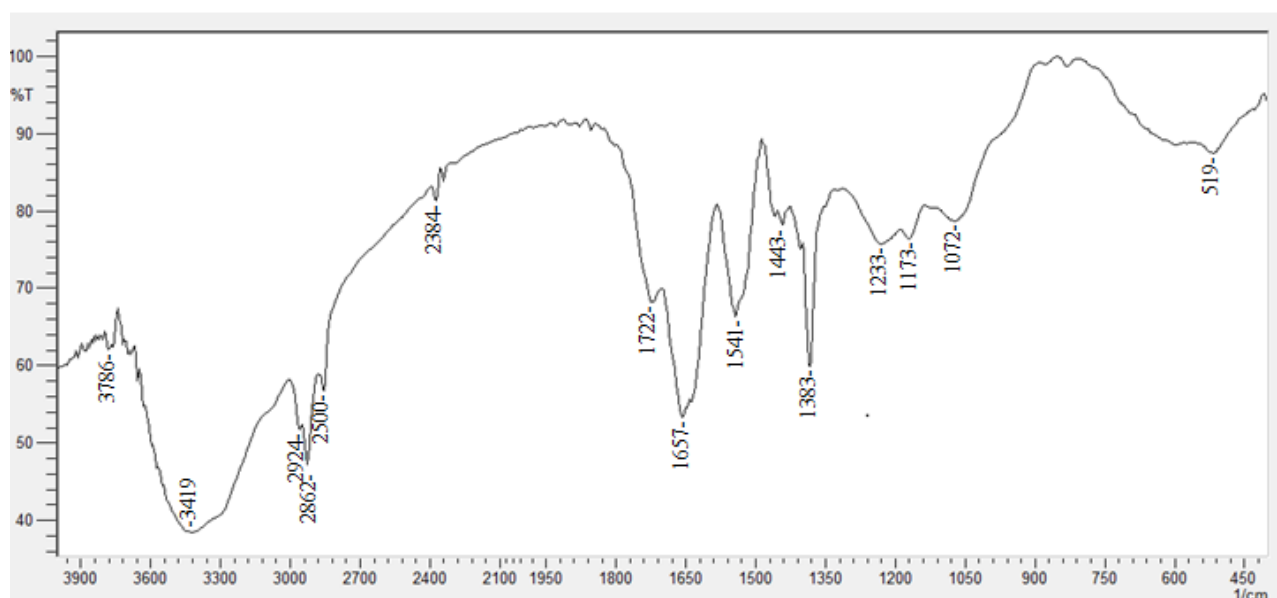
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0.005

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

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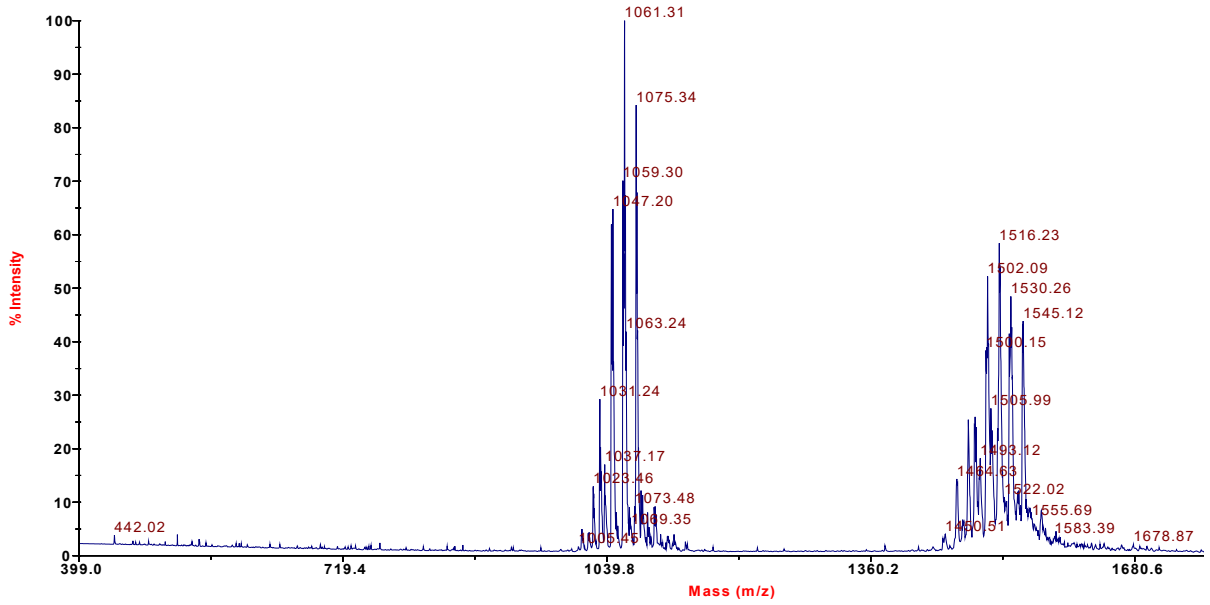
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483 **Figure 2:** FT-IR spectrum of the biosurfactant produced by *Alcaligenes aquatilis* strain
 484 YGD 2906

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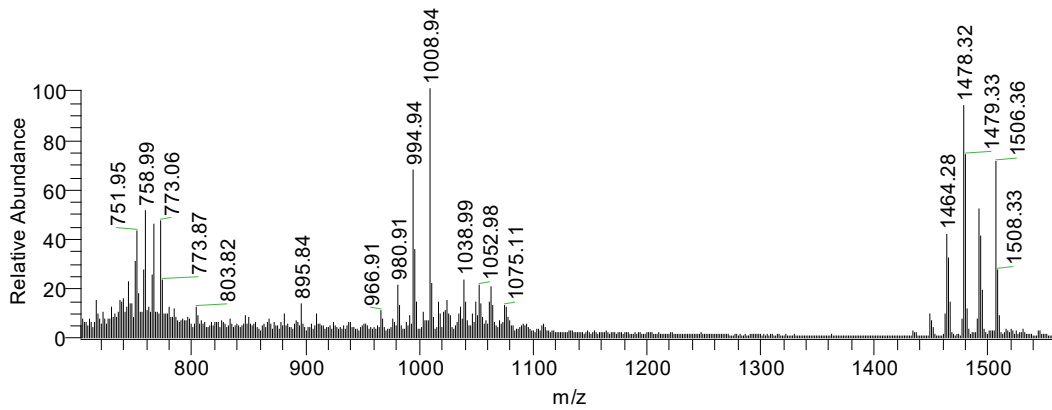


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489 **Figure 3:** MALDI-TOF/MS spectra of lipopeptide sample

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Lipopeptide #390-2240 RT: 6.92-41.20 AV: 1851 NL: 3.29E5
F: + c ESI Full ms [100.00-2000.00]



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493 **Figure 4:** ESI-full MS of lipopeptide sample from LC separation

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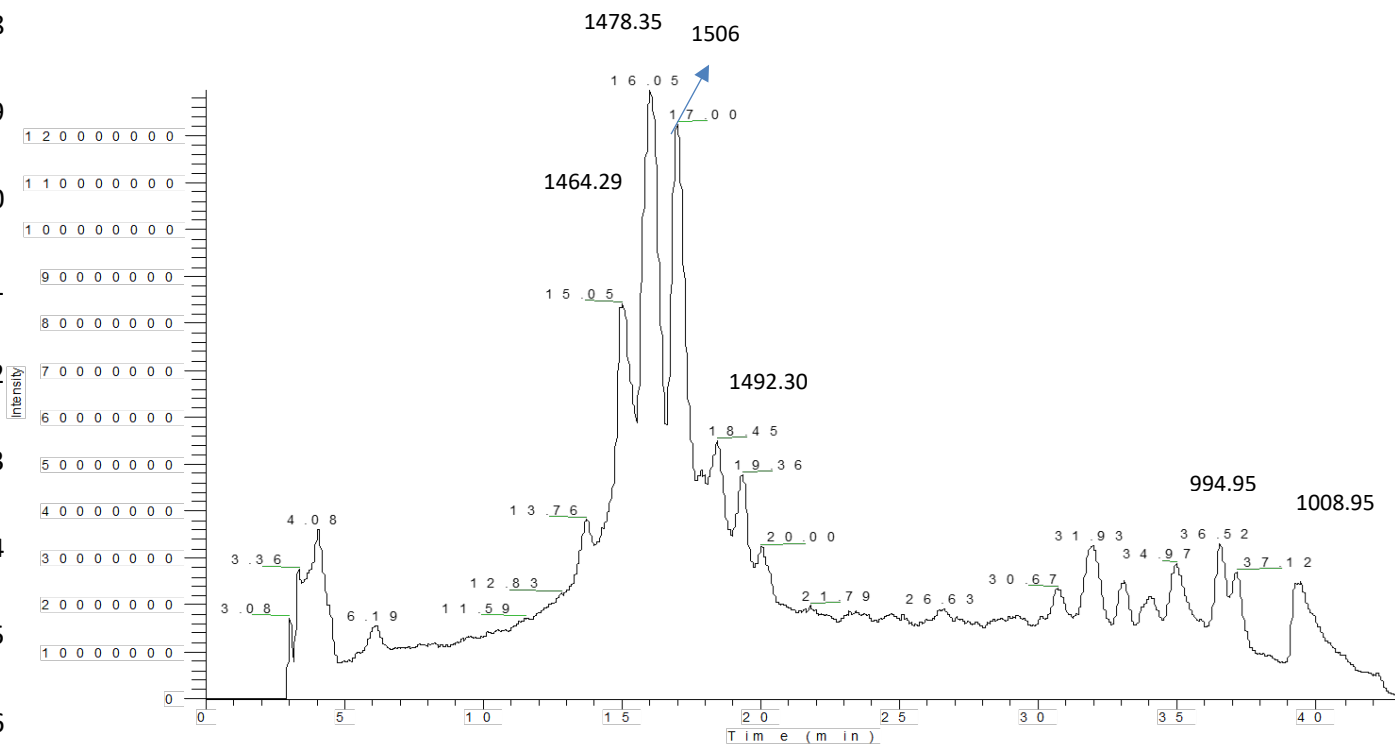
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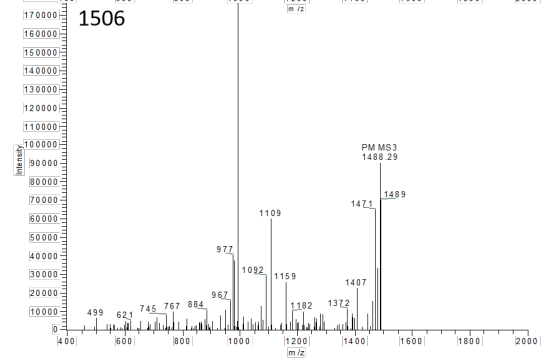
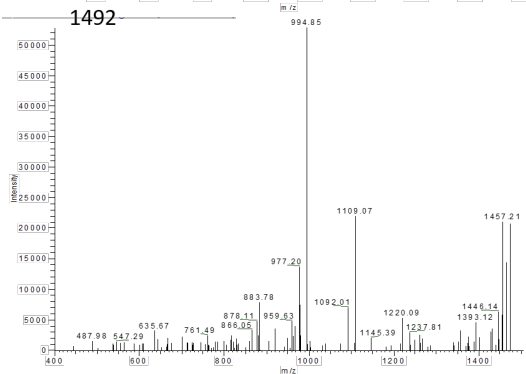
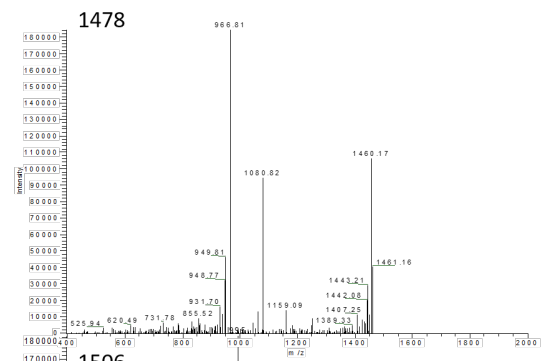
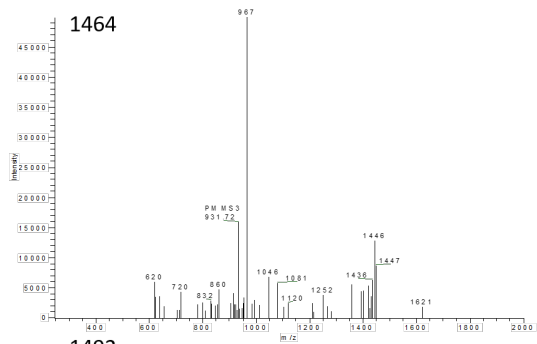
508 **Figure 5:** Chromatogram of Lipopeptide sample

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514 **Figure 6:** LC-MS/MS analysis of ions 1464, 1478, 1492 and 1506

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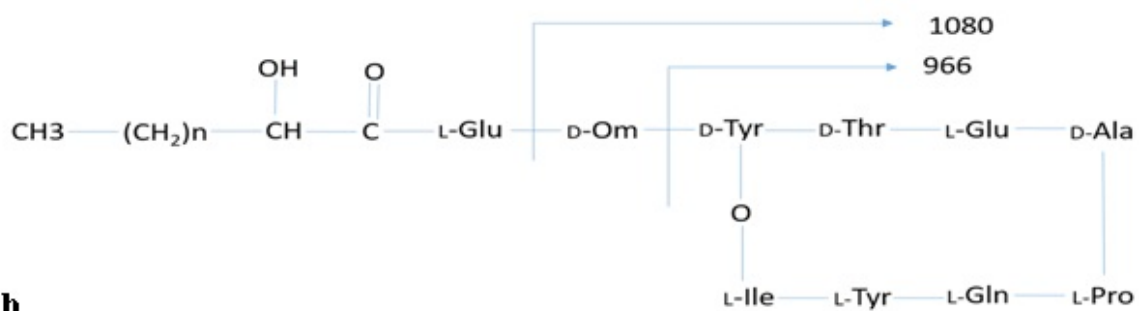
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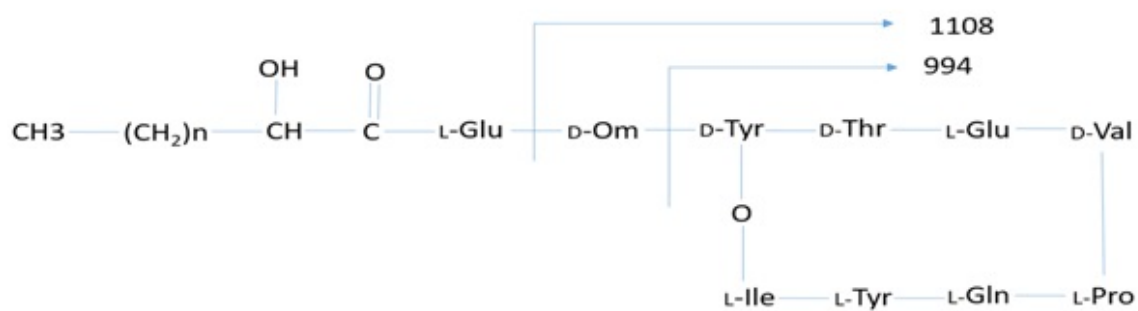
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529 **Figure 7:** Structures of Fengycin A (M+H)⁺(a) and Fengycin B (M+H)⁺ (b) showing the
530 different daughter ions observed in the MS/MS

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