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Abstract

Bacillus methylotrophicus DCS1 strain was isolated from diesel contaminated soil and screened for its ability to produce biosurfactants; it was found to be a potent producer. The structural characterization of the isolated lipopeptides was studied by a variety of analytical techniques. The organic extract of DCS1 lipopeptides was fractionated by silica gel column chromatography (60 Mesh). Fractions containing lipopeptides were collected and identified by tandem mass spectrometry MALDI-TOF-MS and MALDI-TOF MS2. The crude biosurfactants contains a mixture of homologous lipopeptides with molecular weights between 1016 and 1556 Da. Mass spectrometry analysis of partially purified lipopeptides revealed that it contains different isoforms belonging to three families: surfactin, iturin and fengycin. To identify lipopeptides isoforms, MALDI-TOF MS2 was used and ions representing characteristic fragmentations were detected. The mass spectrometry characterization revealed the presence of four variants of surfactin lipopeptides, four variants of pumilacidin that differ according to the β-hydroxy fatty acid chain length as well as the type of amino acid at position 7, five variants of iturin A/mycosubtilin varying in the β-amino fatty acid chain length from C12 to C16, C16 iturin C1, five isoforms of bacillomycin D varying in the β-amino fatty acid chain length from C14 to C18, and six fengycin isoforms that differ according to the length of the β-hydroxy fatty acid side chain as well as the amino acid at position 6. The capacity of B. methylotrohicus DCS1 strain to produce many lipopeptides isoforms belonging to different families and having a structural diversity is a very interesting characteristic that allows them to be used in various fields of biotechnology applications.

Keywords	Bacillus methylotrophicus DCS1; MALDI-TOF-MS; MALDI-TOF MS2; Structure characterization; Surfactin; Iturin; Fengycin.

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Structural characterization and identification of cyclic lipopeptides produced by *Bacillus methylotrophicus* DCS1 strain

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Abstract

Bacillus methylotrophicus DCS1 strain was isolated from diesel contaminated soil and screened for its ability to produce biosurfactants; it was found to be a potent producer. The structural characterization of the isolated lipopeptides was studied by a variety of analytical techniques. The organic extract of DCS1 lipopeptides was fractionated by silica gel column chromatography (60 Mesh). Fractions containing lipopeptides were collected and identified by tandem mass spectrometry MALDI-TOF-MS and MALDI-TOF MS^2. The crude biosurfactants contains a mixture of homologous lipopeptides with molecular weights between 1016 and 1556 Da. Mass spectrometry analysis of partially purified lipopeptides revealed that it contains different isoforms belonging to three families: surfactin, iturin and fengycin. To identify lipopeptides isoforms, MALDI-TOF MS^2 was used and ions representing characteristic fragmentations were detected. The mass spectrometry characterization revealed the presence of four variants of surfactin lipopeptides, four variants of pumilacidin that differ according to the β-hydroxy fatty acid chain length as well as the type of amino acid at position 7, five variants of iturin A/mycosubtilin varying in the β-amino fatty acid chain length from C_{12} to C_{16}, C_{16} iturin C1, five isoforms of bacillomycin D varying in the β-amino fatty acid chain length from C_{14} to C_{18}, and six fengycin isoforms that differ according to the length of the β-hydroxy fatty acid side chain as well as the amino acid at position 6. The capacity of *B. methylotrohicus* DCS1 strain to produce many lipopeptides isoforms belonging to different families and having a structural diversity is a very interesting characteristic that allows them to be used in various fields of biotechnology applications.

Keywords: *Bacillus methylotrophicus* DCS1; MALDI-TOF-MS; MALDI-TOF MS^2; Structure characterization; Surfactin; Iturin; Fengycin.

1. Introduction

Historically, lipopeptides groups were discovered from *Bacillus* species over the years 1950 and 1960. More than 30 different peptides related to different fatty acid chains and more than 100 different substances have been described since then by Jacques [1]. The lipopeptides produced by gram-positive strains have been classified into various types based on their amino acid composition and fatty acid chain length [2]. Three different families of non-ribosomal lipopeptides: surfactins, iturins and fengycins were identified in *Bacillus* spp. between 1949 and 1986 [1]. A new family named kurstakins was identified in *Bacillus thuringiensis* at the year 2000 [3].

Surfactin family is composed of about 20 different lipopeptides [4]. The operon *srfA* and enzymes responsible for the biosynthesis of surfactin were first described for a lipopeptide synthesized by *Bacillus subtilis* [5,6]. They are heptapeptides interlinked with β-hydroxy fatty acid chain length from C_{13} to C_{16} [7]. Surfactin is one of the most powerful biosurfactant known to act as a detergent on biological membranes [8,9]; it has been characterized as an antibacterial, antiviral and anti-mycoplasma agent [10,11].

Iturins are produced by *B. subtilis* and other closely related bacilli [12]. These molecules are composed of cyclic heptapeptide acylated with β-amino fatty acid with chain length from C_{14} to C_{17}. Iturin family includes iturin A and C1, bacillomycin D, F and L and mycosubtilin [13]. Iturins lipopeptides are neutral or mono-anionic. In the different members of iturin family, amino acids in heptapeptides differ slightly. Iturin A is the most known member, it was isolated from *B. subtilis*, which is a strain from soil in Ituri (Zaire) during the year 1957 [14]. Among all the iturins, iturin A has been found to be the most potent antifungal lipopeptide [15,16]. Iturin A reduces the surface tension of water to 54 mN/m and it has the ability to form foam and stabilize it [17]. The members of iturin family as mycosubtilin

present a potent antifungal and hemolytic activities, while their antibacterial activity is limited [18].

At 1968, German and Japanese teams discovered simultaneously a third family of lipopeptides named fengycin, produced by *B. subtilis* and plipastatin produced by *B. cereus* [19,20]. The operon encoding fengycin-plipastatin synthetase was first described in *B. subtilis* 168 in 1997 [21]. Fengycins were known as antifungal molecules and plipastatins as phospholipase A2 inhibitor. Lipopeptides belonging to fengycin family are composed of 10 amino acid residues, this peptide fragment is bounded to a β-hydroxy fatty acid with a side chain length from C_{14} to C_{18}, which could be saturated or unsaturated [22,4]. Fengycins molecules are less hemolytic than iturins and surfactins but retaining a strong antifungal activity, in particular against filamentous fungi [23,24,19].

In this study, we investigated the structural characterization of lipopeptides produced by *B. methylotrophicus* DCS1 strain using a tandem mass spectrometry method.

2. Material and methods

2.1. Bacterial strain and lipopeptides production

Biosurfactant-producing strain used in this study was isolated from diesel contaminated soil in Sfax City, Tunisia. It was selected on the basis of the high hemolytic activity and decreasing surface tension of the culture medium. This strain was identified as *Bacillus methylotrophicus* DCS1 based on its biochemical and 16S rDNA gene sequence analysis [25]. The bacterial strain was maintained at 4 °C and also preserved in glycerol at -80 °C.

B. methylotrophicus DCS1 was inoculated into a 250 ml shake flask containing 25 ml Luria-Bertani (LB) broth medium (g/l): peptone, 10.0; yeast extract, 5.0; and NaCl, 5.0; pH 7.0; and cultivated at 37 °C with shaking at 200 rpm for 18 h as inoculums. A 3% (v/v) of inoculum [$OD_{600\ nm}$ = 7.6] was transferred into a 2 l Erlenmeyer flask containing 250 ml of

Landy medium [26] which contains: glucose 20 g/l, L-glutamic acid 5 g/l, yeast extract 1 g/l, K_2HPO_4 1 g/l, $MgSO_4$ 0.5 g/l, KCl 0.5 g/l, $CuSO_4$ 1.6 mg/l, $Fe_2(SO_4)_3$ 0.4 mg/l, $MnSO_4$ 1.2 mg/l. The initial pH was adjusted to 7.0 and culture was incubated for 72 h at 30 °C with shaking at 150 rpm under sterile conditions.

2.2. Lipopeptides recovery

The culture broth was centrifuged at 8,000 rpm and 4 °C for 20 min to discard the cells. The cell free supernatant was acidified to pH 2.0 by adding 6 N HCl and incubated overnight at 4 °C with agitation for the precipitation of lipopeptides product. The precipitated lipopeptides were then collected by centrifugation at 8,000 rpm, for 20 min at 4 °C, suspended in distilled water and the pH was adjusted to 8.0 with 1.0 N NaOH. The crude lipopeptides obtained was lyophilized [27].

2.3. Amino acid composition determination

The crude lipopeptides (4 mg) was hydrolyzed in 1 ml 6 M HCl at 110 °C overnight in a sealed tube. The amino acids were then analyzed by HPLC, using the Waters AccQTag pre-column derivatization method [28]. Reaction of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent (AQC) yields derivatives that are detected at 254 nm. The analysis was performed on a Nova-Pak C18 column (3.9×150 mm) at a flow rate of 1 ml/min at 37 °C, attached to a Delta 600 chromatographic system with a 2478 Dual Absorbance detector and a 717 plus Auto sampler (Waters, Bedford, MA).

2.4. Extraction and fractionation of lipopeptides

From acid precipitated product, extraction of lipopeptides was performed several times with tetrahydrofuran (THF) solvent. The organic phases recuperated were combined and concentrated in a rotary vacuum evaporator (Büchi, Switzerland).

This extract was tested by thin layer chromatography (TLC) on silica plates 60 F (Merck, Macherel-Nagel) with the mobile phase: chloroform/methanol/water (65:25:4), staining was carried out with phosphomolybdic acid specific for fatty acid moiety and o- Tolidine specific for amino acid moiety, to detect the spots showing the presence of both amino acid and fatty acid parts.

Lipopeptides organic extract was chromatographed on a silica gel column (60 Mesh). Elution was carried out with the same solvent of migration used in TLC and fractions of 1 ml were collected and tested by thin layer chromatography. The fractions collected showing the presence of both amino acid and fatty acid parts were analyzed by tandem mass spectrometry MALDI-TOF-MS and MALDI-TOF MS/MS.

2.5. Matrix-assisted laser desorption ionization time of flight mass spectrometry

The molecular weight of the components of the surfactants was determined by negative- and positive-ion mode electrospray ionization (ESI) analyses (LC/MSD-TOF, Agilent Technologies, Palo Alto, CA). The capillary voltage was 4 kV and 3.5 kV for the positive and negative modes, respectively, with nitrogen as the nebulizing and drying gas. Tandem mass spectrometry (4700 Plus MALDI TOF/TOF, ABSciex, Dublin, CA) was used in the experiment. The full mass spectrum was acquired in the reflector positive-ion mode for the lipopeptides, using dihydroxybenzoic acid (DHB) as the matrix. Subsequent fragmentation of the observed ions was obtained by positive MS/MS analysis.

3. Results and discussion

3.1. Amino acid composition of crude lipopeptides DCS1

The amino acid content of crude lipopeptides DCS1 was determined by analysis of a hydrolyzed sample followed by derivatization using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate reagent (AQC). The pairs Glu/Gln and Asp/Asn cannot be determined by this technique as hydrolysis of the peptide converts Gln and Asn amino acids into Glu and Asp. The sample of the crude lipopeptides DCS1 contains about 40% proteins.

A variety of amino acids was detected in crude lipopeptides DCS1, the majority of them are present in the composition of lipopeptides belonging to surfactin, iturin and fengycin families, they are: Glx, Asx, Tyr, Leu, Pro, Ser, Val, Ala and Ile with molar ratios of 19.5%; 14.74%; 8.45%, 8.33%, 7.49%, 6.29%, 5.69%, 4.49% and 3.12%, respectively (Table 1).

The amino acid Glu is present with 1 residue in the composition of surfactin, pumilacidin and bacillomycin D and with 2 residues in the composition of fengycin lipopeptides, while Gln amino acid is present with 1 residue in the composition of iturin and fengycin lipopeptides families. The amino acid Asp is present with 1 residue in the composition of surfactin and pumilacidin, while the amino acid Asn is present with 2 residues in the composition of bacillomycin D and with 3 residues in iturin lipopeptides. Tyr and Pro amino acids are present in the composition of iturin and fengycin lipopeptides families. The amino acid Leu is present with 3 or 4 residues in surfactin molecules and with 4 residues in pumilacidin molecules. The amino acid Ser is present in the composition of lipopeptides belonging to iturin family, while Val, Ala and Ile amino acids are present in the composition of lipopeptides belonging to surfactin and fengycin families. This result can give an idea about the type of lipopeptides produced.

3.2. Characterization of lipopeptides extract by TLC

The characterization of DCS1 extract by TLC showed many spots at different levels of migration, the same spots appeared when we used phosphomolybdic acid, iodine vapor and ultraviolet light which correspond to different lipopeptides molecules (Fig. 2).

3.3. Detection of lipopeptides by mass spectrometry MALDI-TOF analysis

3.3.1. Identification and characterization of surfactin lipopeptides by tandem mass spectrometry

Mass spectrometry analysis of partially purified lipopeptides DCS1, fractionated by silica gel column, show the presence of four well-resolved clusters of peaks, the first within the mass range *m/z* 1016.7 and 1058.8 Da (Fig. 3A), eluted in silica gel column at the first fractions collected from F1 to F36. By comparing the mass (*m/z*) with the mass numbers reported for lipopeptides synthesized by other *Bacillus* strains, the precursor ions at *m/z* 1016.7, 1030.7, 1044.7 and 1058.8 (Fig. 3A) were assigned as the sodium ion adducts of homologous surfactin lipopeptides with 993.7, 1007.7, 1021.7 and 1035.8 Da mass, respectively. The structure of surfactin lipopeptides was confirmed by MS/MS fragment analysis.

MALDI-TOF MS^2 analysis was used to do the fragmentation of lipopeptides to obtain more precise information on their chemical structure. The fragmentation patterns of the parent ions at *m/z* 1016.7, 1030.7, 1044.7 and 1058.8, reported in Fig. 4, show fragments that can correspond to differences among some amino acids in the peptide moiety and the fatty acid.

For the spectra of surfactin, the fragmentation pattern of the sodiated molecule $[M + Na]^+$ at *m/z* 1016.7 was illustrated in Fig. 4a; the MS/MS spectrum of the parent ion at *m/z* 1016.7 shows a set of daughter ions. The fragmentation resulted in the appearance of product ions at *m/z* 917.6 $[M + Na - (Val = 99 Da)]^+$, 804.5 $[M + Na - (Val + Leu = 212 Da)]^+$ and 786.5 $[M + Na - (Val + Leu + H_2O = 230 Da)]^+$. The obtained results indicated that the main parent ion

206 at *m/z* 1016.7 corresponds to surfactin, a cyclic lipopeptide with Val residue at position 4 and
207 7 and a fatty acid chain of 13 carbons. Our result is in accordance with that of Vater et al. [29]
208 which reported that a main parent ion at *m/z* 1016.7 was attributed to a small amount of the
209 sodium adduct of a valine-7 surfactin identified from *B. subtilis* C-1.

210 The fragmentation pattern of the sodiated molecule $[M + Na]^+$ at *m/z* 1030.7 was
211 illustrated in Fig. 4b. The fragmentation resulted in the appearance of two product ions series
212 deriving from the initial opening of the lactone ring. The first series contains the product ions
213 from the fragmentation of surfactin precursor ion from the C-terminal carboxylate group of
214 the aliphatic peptide moiety. The values at *m/z* 917.6, 804.5 and 786.5 correspond
215 respectively, to the losses of Leu/Ile (−113 Da), Leu/Ile-Leu (−226 Da) and Leu/Ile-Leu-H_2O
216 (−244 Da) from the parent ion *m/z* 1030.7. Therefore, the amino acid at position 7 is Leu/Ile.
217 The second series contains the product ions from the fragmentation of surfactin precursor ion
218 on the side of the fatty acid chain and the N-terminal of the aliphatic peptide moiety. Values
219 at *m/z* 707.5 and 594.4 correspond respectively, to the losses of C_{13} *β*-hydroxy fatty acid
220 chain-Glu (−323 Da) and C_{13} *β*-OH-fatty acid side chain-Glu-Leu (−436 Da) from the
221 precursor ion *m/z* 1030.7 (Table 2). The obtained results indicated that the peak at *m/z* 1030.7
222 corresponds to surfactin, a cyclic lipopeptide with a fatty acid chain of 13 carbons and Leu or
223 Ile residue at position 7.

224 The same fragmentation sites were observed with the sodiated molecules $[M + Na]^+$ at
225 *m/z* 1044.7 and 1058.8. The fragmentation pattern of the peak 1044.7 was illustrated in Fig.
226 4c. The fragmentation resulted in the appearance of product ions from the C-terminal tail of
227 the aliphatic peptide moiety at *m/z* 931.6, 818.5, 800.5 and 604.4 corresponding respectively,
228 to the losses of Leu/Ile (−113 Da), Leu/Ile-Leu (−226 Da), Leu/Ile-Leu-H_2O (−244 Da) and
229 Leu/Ile-Leu-Asp-Val (−440 Da) from the parent ion *m/z* 1044.7. Therefore, the amino acids at
230 position 7 and 4 are Leu/Ile and Val, respectively. Other product ions resulted from the

fragmentation on the side of the fatty acid chain and the N-terminal tail, *m/z* observed were 707.5, 594.4, 481.3 and 463.3 corresponding respectively, to the losses of C_{14} *β*-hydroxy fatty acid chain-Glu (−337 Da), C_{14} *β*-OH-fatty acid side chain-Glu-Leu (−450 Da), C_{14} *β*-OH-fatty acid side chain-Glu-Leu-Leu (−563 Da) and C_{14} *β*-OH-fatty acid side chain-Glu-Leu-Leu-H_2O (−581 Da) from the precursor ion *m/z* 1044.7 (table 2). The obtained results indicated that the peak at *m/z* 1044.7 corresponds to surfactin, a cyclic lipopeptide with a fatty acid chain of 14 carbons and Leu or Ile residue at position 7. Alajlani et al. [30] identified from *B. subtilis* strain BIA, C_{14} surfactin [Leu7/Ile7] $[M + H]^+$ with an *m/z* ratio 1022.7.

The same fragmentation model was observed for the parent ion *m/z* 1058.8 (table 2) and results showed that this lipopeptide correspond to C_{15} surfactin [Leu7/Ile7]. Product ions were illustrated in Fig. 4d. Our results of fragmentation of the parent ions *m/z* 1030.7, 1044.7 and 1058.8 are in accordance with those of Pecci et al. [16] which characterize lipopeptides produced by *B. licheniformis* V9T14 strain using LC/ESI-MS/MS.

To conclude, *B. methylotrophicus* produce four surfactin isoforms which are C_{13} surfactin [Val7] and from C_{13} to C_{15} surfactin [Leu7/Ile7].

3.3.2. Identification and characterization of pumilacidin isoforms

The second cluster of peaks at *m/z* values between 1036.6 and 1093.7 Da (Fig. 3B), eluted at the fractions collected from F37 to F48 contains pumilacidin and iturin isoforms.

The precursor ions at *m/z* 1036.6, 1050.6, 1064.6, and 1078.7 could be attributed to hydrogen ion adducts of pumilacidin isoforms. The peaks of pumilacidin lipopeptides at *m/z* 1036.6, 1050.6 and 1064.6 show ambiguities in the structure of the molecules, they could be assigned to C_{15}, C_{16} and C_{17} pumilacidin [Val7], respectively or to C_{14}, C_{15} and C_{16} [Ile7], respectively. Pabel et al. [31] determined the structure analysis of *m/z* 1050.8 $[M + H]^+$ by PSD MALDI MS and identified it as a pumilacidin containing a β-hydroxy fatty acid with a

chain length of 16 carbon atoms which is isolated from *Bacillus* isolates. Therefore, the peak at *m/z* 1050.6 consists of protonated C_{16} pumilacidin [Val7]. According to Pabel et al. [31], the mass peaks 1058.9, 1072.9 and 1086.9 were assigned, respectively, to sodiated C_{15}, C_{16} and C_{17} pumilacidin. Consequently, *m/z* 1036.6, 1050.6 and 1064.6 could be attributed to protonated C_{15}, C_{16} and C_{17} pumilacidin [Val7], respectively. The peak at *m/z* 1078.7 corresponds to protonated C_{17} pumilacidin [Ile7].

3.3.3. Identification and characterization of iturin family lipopeptides

The values of *m/z* 1053.6 and 1067.6 (Fig. 3B) could correspond to C_{14} and C_{15} $[M + Na]^+$ bacillomycin D described in previous report [11]. The identification of these isoforms of bacillomycin D was reported by Lee et al. [32] and Cao et al. [33] who showed molecular ion peaks at *m/z* 1031.6 and *m/z* 1045.6 identified in *Bacillus* sp. LM7 and *B. subtilis* SQR 9, respectively, as C_{14} and C_{15} bacillomycin D $[M + H]^+$, respectively. In the spectrum (Fig. 3B) we can also see a small peak at *m/z* 1031.6 which corresponds to C_{14} bacillomycin D $[M + H]^+$.

Fig. 3B shows *m/z* values of 1079.7 and 1093.7 Da, which could be attributed to sodium ion adducts of C_{15} iturin A / C_{15} mycosubtilin or C_{14} bacillomycin F and C_{16} iturin A / C_{16} mycosubtilin or C_{15} bacillomycin F, respectively. According to Pabel et al. [31], the mass peaks 1079.7 and 1093.7 were assigned, respectively, to sodiated C_{15} and C_{16} iturin isolated from *Bacillus* isolates. Pathak and Keharia [34] reported that *Bacillus subtilis* K1 strain produce iturin homologues and the precursor ions at *m/z* 1079.5 and 1093.5 were assigned as sodium adducts of C_{15} and C_{16} iturin A, respectively. So, we can assign these two masses to sodiated C_{15} and C_{16} iturin A.

The molecular ion peak at 1092.7 could be assigned to sodium ion adduct of iturin C1 (Fig. 3B), our result is in accordance with that of Alajlani et al. [30] who reported that the

peak with *m/z* of 1070.6 corresponds to the mass of $[M + H]^+$ ion of C_{16} iturin from *B. subtilis* strain BIA.

The third cluster of peaks in the range 1037.6-1111.6 Da (Fig. 3C) and the fourth in the range 1485.9-1557.9 Da (Fig. 3D), were eluted in silica gel column at the fractions collected from F53 to F66. Based on published literature [15,29,35,36], the precursor ions at *m/z* values of 1037.6, 1051.6 and 1065.6 were assigned as the sodium ion adducts of iturin isoforms corresponding to iturin A / mycosubtilin lipopeptides varying in the β-amino fatty acid chain length from C_{12} to C_{14}. Therefore, *m/z* values of 1037.6, 1051.6, 1065.6, 1079.7 and 1093.7 are attributed to the sodium ion adducts of iturin A / mycosubtilin isoforms from C_{12} to C_{16} β-amino fatty acid chain length, respectively.

The high resolution mass *m/z* 1081.6 correspond to C_{16} bacillomycin D $[M + Na]^+$ as reported by Tabbene et al. [11] and *m/z* 1095.7 correspond to C_{17} bacillomycin D $[M + Na]^+$ as reported by Lee et al. [32] which showed that molecular ion peak at *m/z* 1073.5883 was identified as C_{17} bacillomycin D $[M + H]^+$.

Gong et al. [37] identified four homologous of bacillomycin D with C_{14} to C_{17} fatty acid chain with protonated molecular ion peaks of 1031.8, 1045.9, 1059.7 and 1073.9, respectively. Therefore, *m/z* values of 1053.6, 1067.6 1081.6, 1095.7 and 1109.6 could be assigned as the sodium ion adducts of bacillomycin D isoforms from C_{14} to C_{18} β-amino fatty acid chain length, respectively, differing by (CH_2 = 14 Da).

3.3.4. Identification and characterization of fengycin lipopeptides by tandem mass spectrometry

The fourth group of peaks with *m/z* values of 1449.9, 1485.9 and 1499.9 (Fig. 3D), could be attributed to C_{15} fengycin A $[M + H]^+$, C_{16} fengycin A $[M + Na]^+$ and C_{17} fengycin A [M +

305 Na]$^+$, respectively, as reported by Cao et al. [33], Wang et al. [38] and Bie et al. [39] who
306 described the production of these fengycin isoforms with hydrogen ionization.

307 The precursor ions at *m/z* values of 1513.9, 1527.9 and 1555.9 Da could be attributed to
308 C_{16} fengycin B [M + Na]$^+$, C_{17} fengycin B [M + Na]$^+$ and C_{19} fengycin B [M + Na]$^+$,
309 respectively (Fig. 3D), they are isoforms of fengycin B varying in β-hydroxy fatty acid chain
310 length. Our results are in accordance with those of Bie et al. [39] who identified C_{16} fengycin
311 B [M + H]$^+$ and C_{17} fengycin B [M + H]$^+$ at *m/z* 1491.7 and 1505.9, respectively from *B.*
312 *subtilis* fmbJ strain.

313 The significant discrimination between fengycins A and B lies in that the amino acid at
314 position 6 of A type is Ala while in B type it is Val. Therefore, lipopeptides DCS1 mixture is
315 composed of six fengycin isoforms that differ according to the length of the fatty acid side
316 chain as well as the peptide amino acid composition.

317 For each group, the different peaks can differ by 14 Da, which indicated that several
318 lipopeptides analogs contain distinct numbers of $–CH_2$- group.

319 The spectrum of fengycin lipopeptides shows two peaks at *m/z* 1499.9 and 1513.9 with
320 ambiguity in the structure of the molecule.

321 The fragmentation of the parent ion at *m/z* 1499.9 [M + Na]$^+$ (Fig. 5a) shows the
322 appearance of the product ion at *m/z* 1102.6 [M + Na - (FA + AA1 = 398 Da)]$^+$, this peak is
323 the result of the loss of a fatty acid chain with 17 carbons and the first amino acid, Glu
324 residue. The product ion at *m/z* 988.5 [M +Na - (FA + AA1 + AA2 = 512 Da)]$^+$ corresponds
325 to the loss of C_{17} fatty acid with Glu and Orn residues. Therefore, the peak at *m/z* 1499.9
326 consists of C_{17} fengycin A. Our result of fragmentation is in accordance with that of Liu et al.
327 [40] and Bie et al. [39] who reported that product ions of *m/z* 1080 and 966 were found in the
328 CID spectrum of precursor ion at *m/z* 1477.5 protonated molecular ion peak [M + H]$^+$ of
329 circular C_{17} fengycin A from *B. amyloliquefaciens* BZ-6 and *B. subtilus* fmbJ strains,

330 respectively. Pecci et al. [16] reported that the protonated molecule at *m/z* 1478 corresponded
331 to C_{17} fengycin A produced by *B. licheniformis* V9T14 strain.

332 The fragmentation pattern of the peak at *m/z* 1513.9 $[M + Na]^+$ was illustrated in Fig. 5b,
333 it resulted in the appearance of the product ion at *m/z* 1130.6 [M + Na - (FA + AA1 = 384
334 Da)]$^+$. This peak is the result of the loss of a fatty acid chain with 16 carbons and the first
335 amino acid, Glu residue. The product ion at *m/z* 1016.5 [M +Na - (FA + AA1 + AA2 = 498
336 Da)]$^+$ corresponds to the loss of C_{16} fatty acid with Glu and Orn residues. Thus, we can
337 conclude that this lipopeptide is C_{16} fengycin B. Our result of fragmentation is in accordance
338 with that of Bie et al. [39] who reported that product ion fragments at *m/z* 1108 and *m/z* 994
339 were obtained in CID spectrum of the protonated molecular ion peak $[M + H]^+$ of the
340 precursor ion at *m/z* 1491.7 corresponding to C_{16} fengycin B from *B. subtilis* fmbJ strain.

341 The results found in this research are in contrast with those published by Qian et al. [41]
342 who reported that the strain *B. methylotrophicus* SHB 114 isolated from South China Sea
343 produced three anti-fungal lipopeptides of the bacillomycin Lc family.

344 Different *Bacillus* strains exhibit diversity in production of cyclic lipopeptides, with most
345 strains reported to produce lipopeptides belonging to only one family while few reported to be
346 co-producers of lipopeptides belonging to two or all the three families [15,16,29,42].

347

348 **4. Conclusion**

349 In this study, a variety of cyclic lipopeptides belonging to surfactin, iturin and fengycin
350 families were isolated from *B. methylotrophicus* DCS1 strain and their structures were
351 elucidated through tandem mass spectrometry. We identified the parent ions of lipopeptides
352 isoforms obtained by MALDI-TOF-MS, then, we defined a MALDI-TOF MS^2 method able to
353 simultaneously characterize the structure and the composition of the produced lipopeptides.
354 We identified 25 lipopeptides variants belonging to the three different families and each

family contains many lipopeptides isoforms differing either by the length of the fatty acid or by the amino acid composition of the peptide cycle. This is an interesting feature which gives importance to *B. methylotrohicus* DCS1 strain for future uses of their produced lipopeptides in biotechnological and pharmaceutical interest.

Acknowledgements

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538
539

Figure captions

Figure. 1 Amino acid composition analysis by HPLC of crude lipopeptides DCS1 hydrolyzed sample

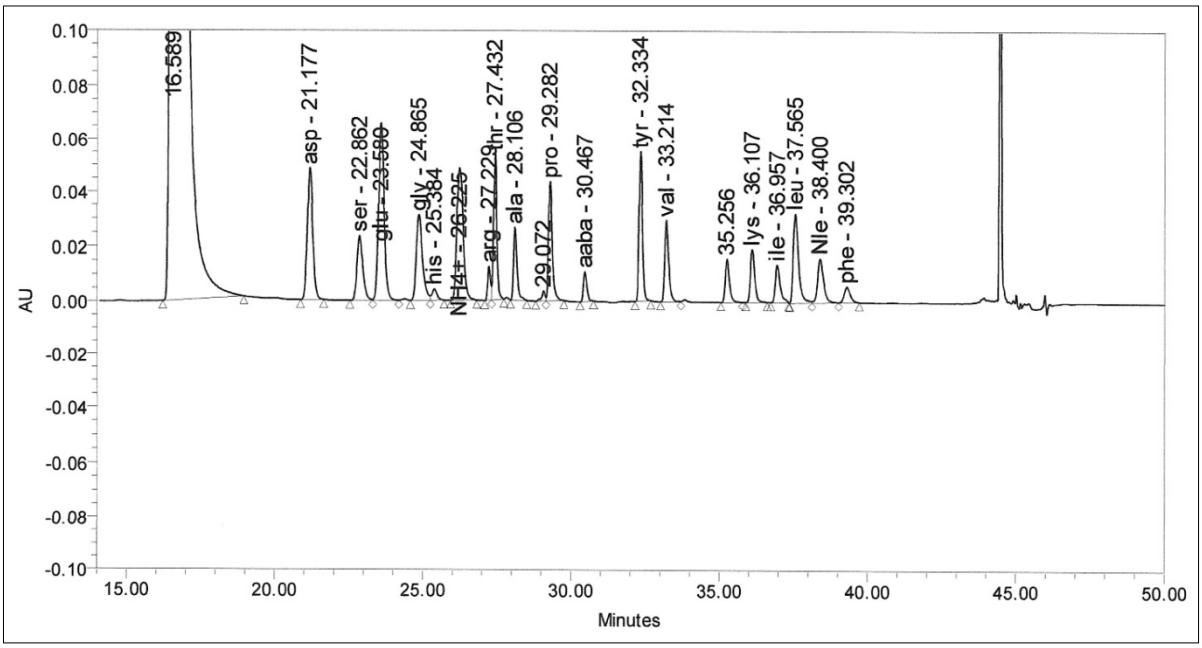
Figure. 2 Thin layer chromatography (TLC) on silica plates 60 F of lipopeptides extract. Revelation with phosphomolybdic acid (a), iodine vapor (b) and ultraviolet light (c)

Figure. 3 Mass spectrometry (MALDI-TOF-MS) analysis of lipopeptides DCS1 molecular masses. Spectra of lipopeptides belonging to surfactin family (A), surfactin and iturin families (B), iturin family (C) and fengycin family (D)

Figure. 4 Product ions spectra obtained by MALDI-TOF-MS/MS of the sodiated molecules $[M + Na]^+$ of surfactin isoforms at *m*/*z* 1016.7 (a) 1030.7 (b) 1044.7 (c) and 1058.8 (d)

Figure. 5 Product ions spectra obtained by MALDI-TOF-MS/MS of the sodiated molecules $[M + Na]^+$ of fengycin isoforms at *m*/*z* 1499.9 (a) and 1513.9 (b)

Fig. 1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asp - 21.177
ser - 22.862
glu - 23.500
gly - 24.865
his - 25.384
NH4+ - 26.225
arg - 27.229
thr - 27.432
ala - 28.106
29.072
pro - 29.282
aaba - 30.467
tyr - 32.334
val - 33.214
35.256
lys - 36.107
ile - 36.957
leu - 37.565
Nle - 38.400
phe - 39.302
AU
Minutes
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00

Fig. 2

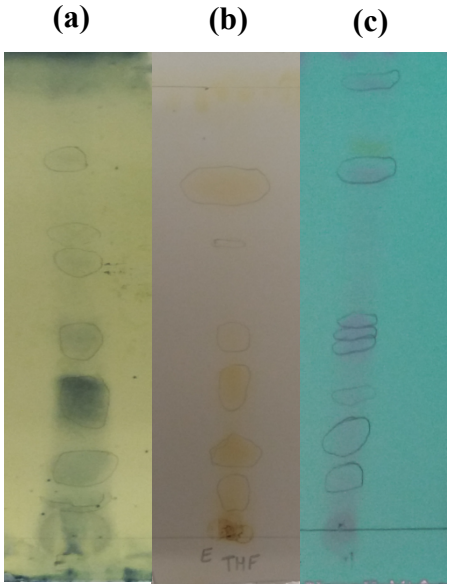
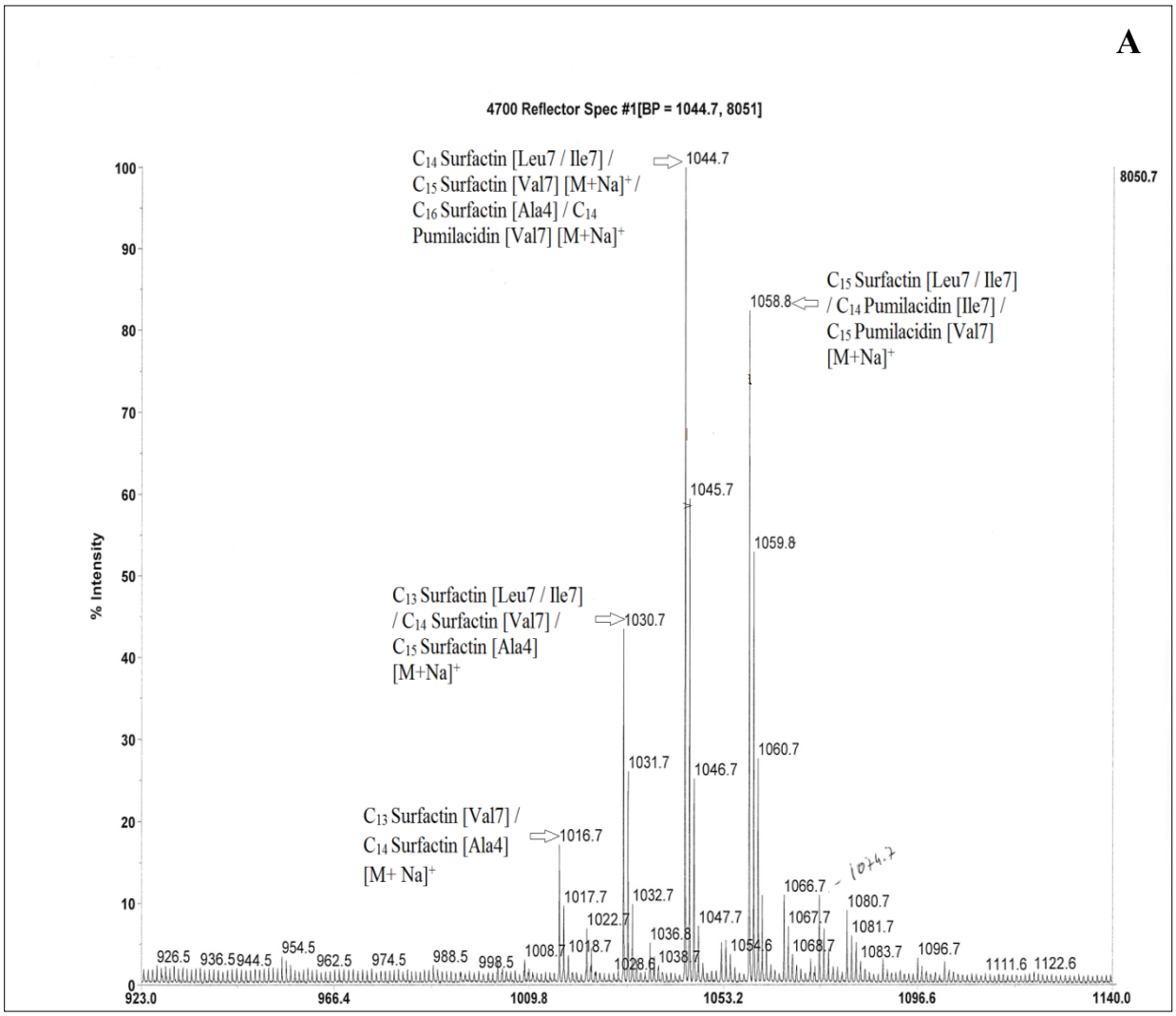
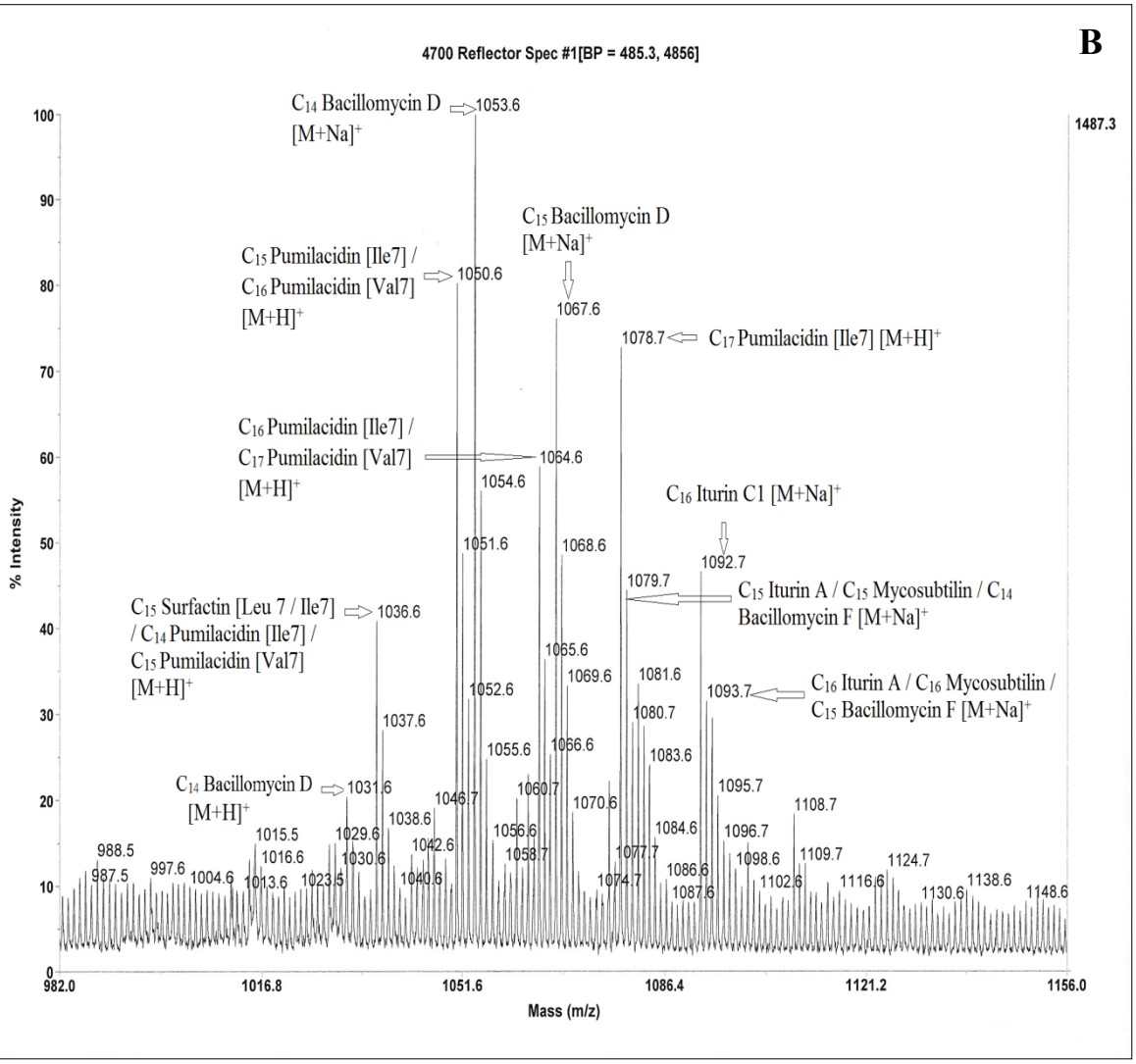
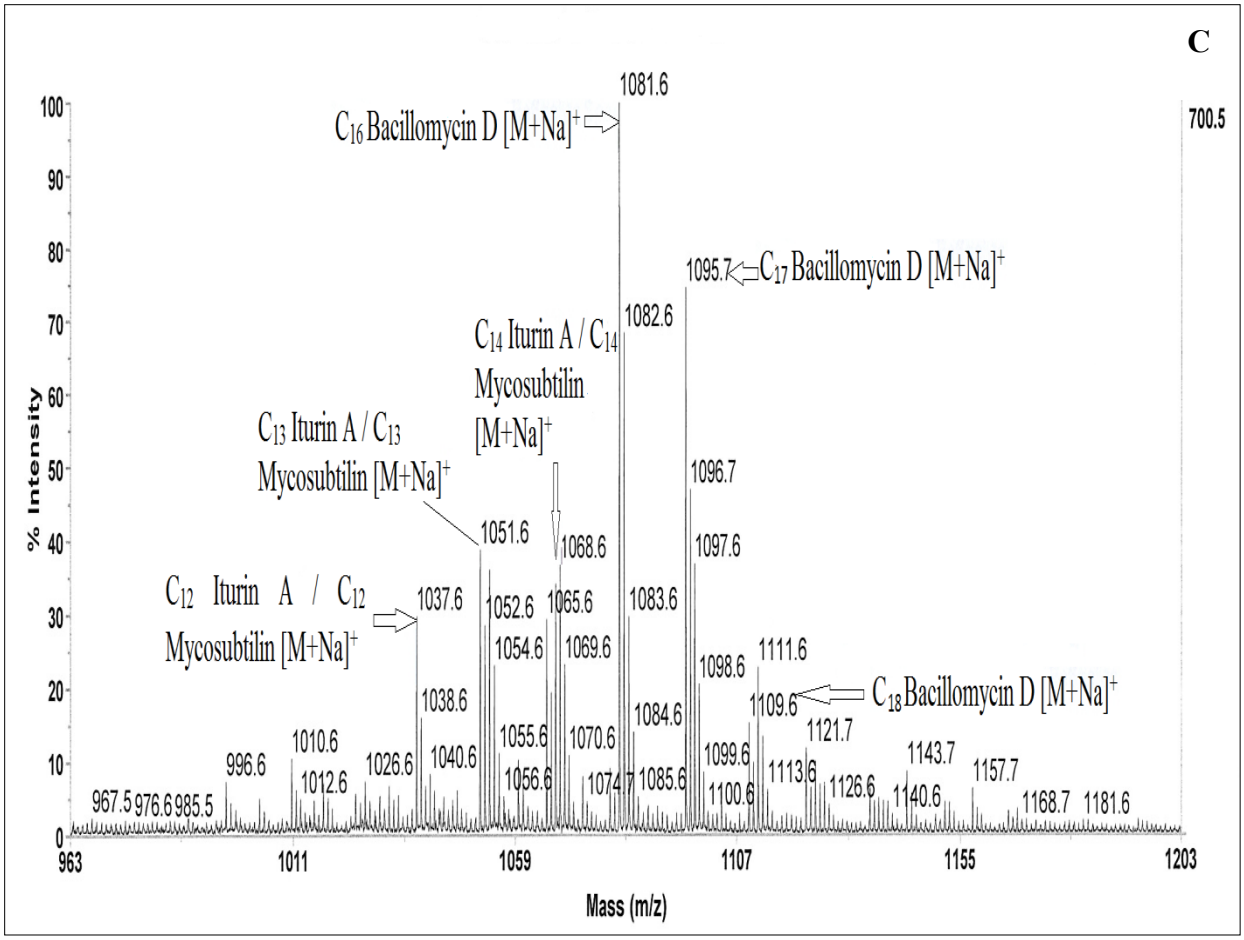


Fig. 3


A
4700 Reflector Spec #1[BP = 1044.7, 8051]
% Intensity
C14 Surfactin [Leu7 / Ile7] / C15 Surfactin [Val7] [M+Na]+ / C16 Surfactin [Ala4] / C14 Pumilacidin [Val7] [M+Na]+
1044.7
8050.7
C15 Surfactin [Leu7 / Ile7] / C14 Pumilacidin [Ile7] / C15 Pumilacidin [Val7] [M+Na]+
1058.8
1045.7
1059.8
C13 Surfactin [Leu7 / Ile7] / C14 Surfactin [Val7] / C15 Surfactin [Ala4] [M+Na]+
1030.7
1031.7
1046.7
1060.7
C13 Surfactin [Val7] / C14 Surfactin [Ala4] [M+ Na]+
1016.7
1017.7
1032.7
1022.7
1047.7
1036.8
1066.7
1080.7
1067.7
1081.7
1068.7
1083.7
1096.7
1111.6
1122.6
926.5
936.5
944.5
954.5
962.5
974.5
988.5
998.5
1008.7
1018.7
1028.6
1038.7
1054.6
923.0
966.4
1009.8
1053.2
1096.6
1140.0

B


4700 Reflector Spec #1[BP = 485.3, 4856]
C14 Bacillomycin D [M+Na]+ 1053.6
1487.3
C15 Bacillomycin D [M+Na]+
C15 Pumilacidin [Ile7] / C16 Pumilacidin [Val7] [M+H]+ 1050.6
1067.6
1078.7 C17 Pumilacidin [Ile7] [M+H]+
C16 Pumilacidin [Ile7] / C17 Pumilacidin [Val7] [M+H]+ 1064.6
1054.6
C16 Iturin C1 [M+Na]+
1092.7
1051.6
1068.6
1079.7
C15 Iturin A / C15 Mycosubtilin / C14 Bacillomycin F [M+Na]+
C15 Surfactin [Leu 7 / Ile7] / C14 Pumilacidin [Ile7] / C15 Pumilacidin [Val7] [M+H]+ 1036.6
1065.6
1069.6
1081.6
1093.7
C16 Iturin A / C16 Mycosubtilin / C15 Bacillomycin F [M+Na]+
1052.6
1080.7
1037.6
1055.6
1066.6
1083.6
C14 Bacillomycin D [M+H]+ 1031.6
1046.7
1060.7
1095.7
1108.7
1070.6
1038.6
1015.5
1029.6
1056.6
1084.6
1096.7
988.5
1016.6
1030.6
1042.6
1058.7
1077.7
1098.6
1109.7
1124.7
987.5
997.6
1004.6
1013.6
1023.5
1040.6
1074.7
1086.6
1102.6
1116.6
1138.6
1087.6
1130.6
1148.6
% Intensity
Mass (m/z)
982.0
1016.8
1051.6
1086.4
1121.2
1156.0


C
1081.6
700.5
C16 Bacillomycin D [M+Na]+
1095.7
C17 Bacillomycin D [M+Na]+
1082.6
C14 Iturin A / C14 Mycosubtilin [M+Na]+
C13 Iturin A / C13 Mycosubtilin [M+Na]+
1096.7
1051.6
1068.6
1097.6
C12 Iturin A / C12 Mycosubtilin [M+Na]+
1037.6
1052.6
1065.6
1083.6
1054.6
1069.6
1098.6
1111.6
1038.6
1109.6
C18 Bacillomycin D [M+Na]+
1084.6
1121.7
1010.6
1055.6
1070.6
1099.6
1143.7
996.6
1012.6
1026.6
1040.6
1056.6
1074.7
1085.6
1113.6
1126.6
1157.7
967.5
976.6
985.5
1100.6
1140.6
1168.7
1181.6
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
963
1011
1059
1107
1155
1203

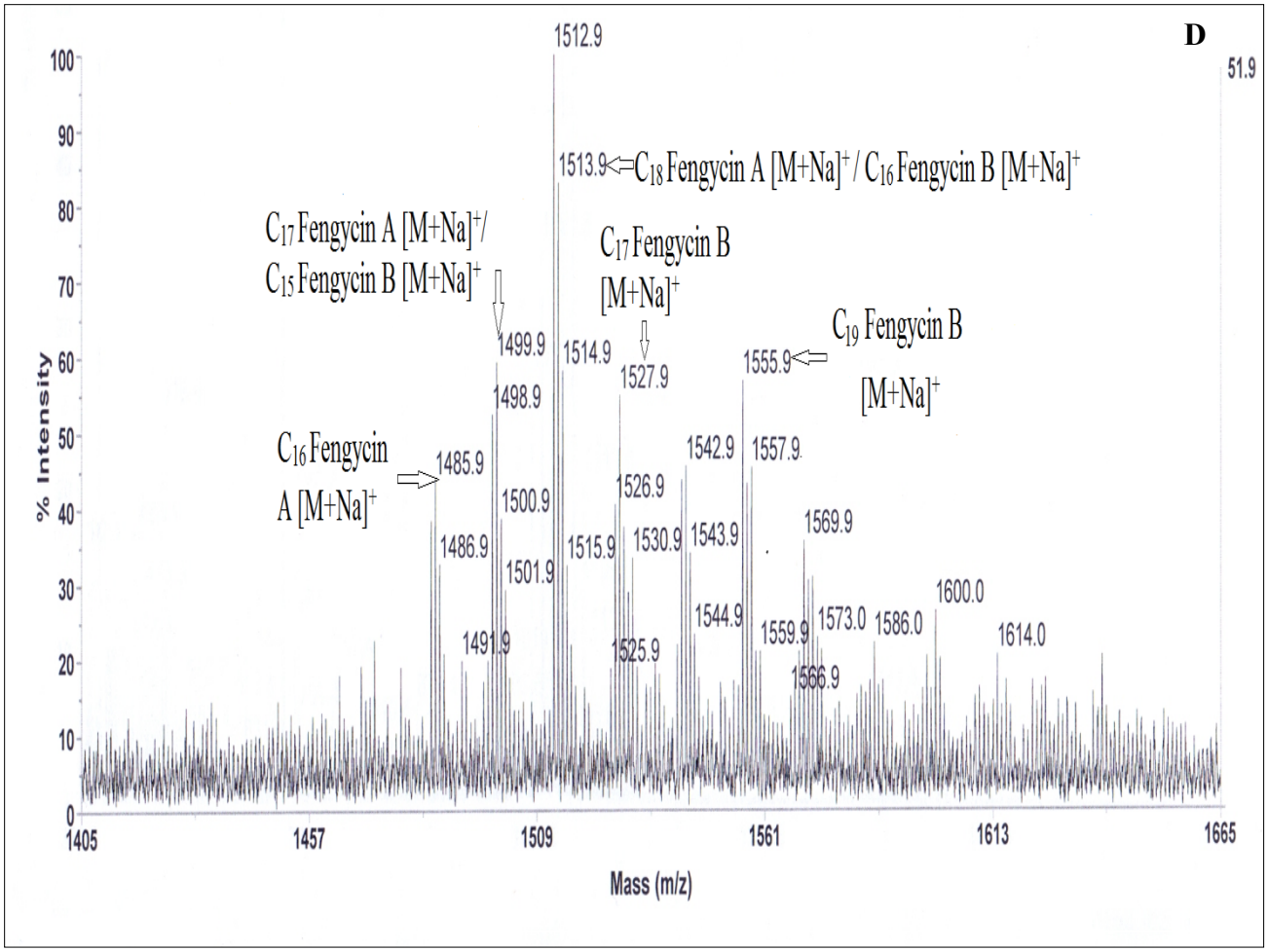
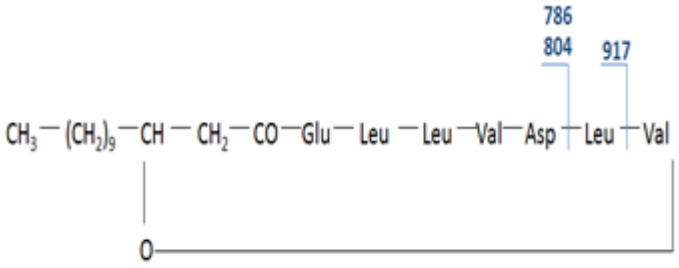
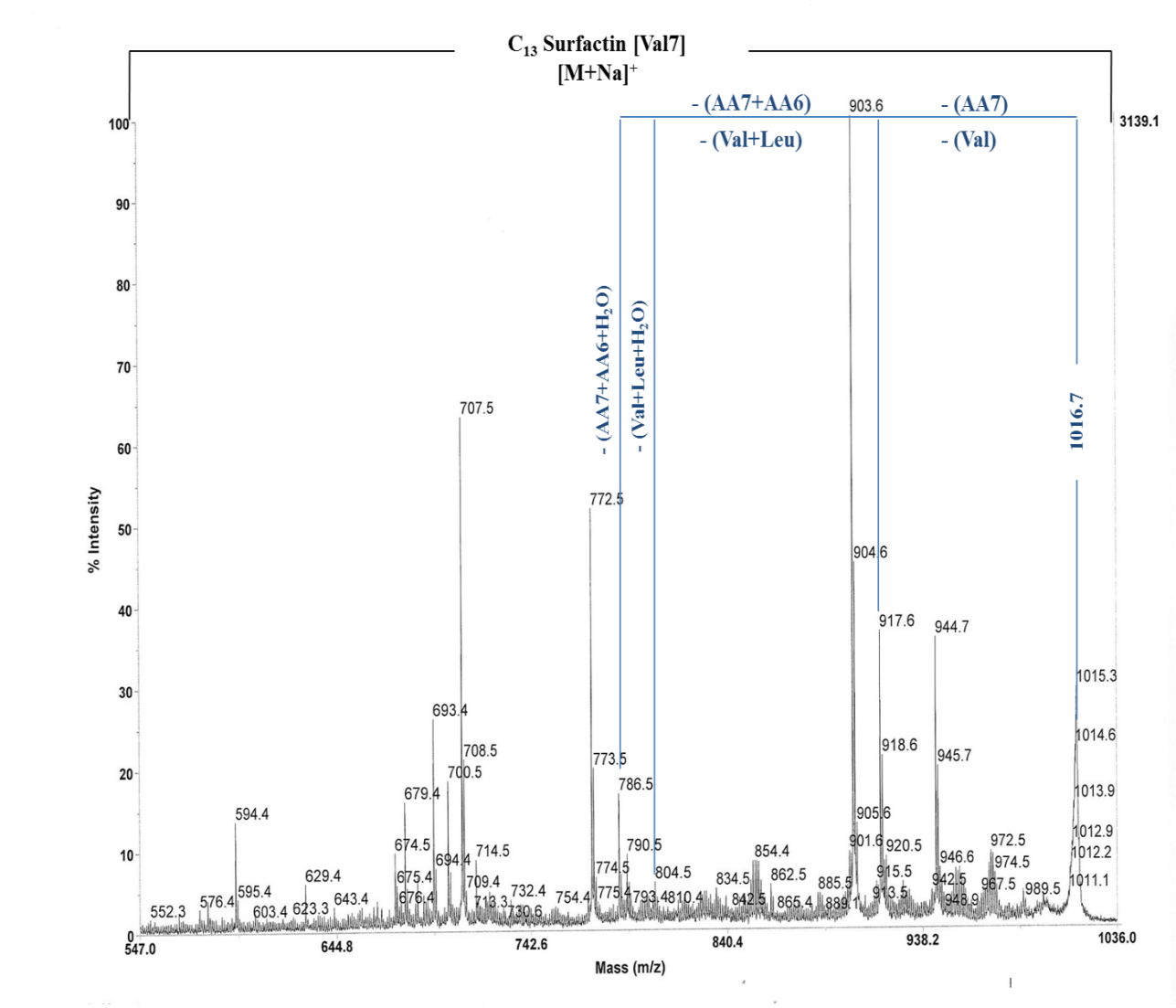
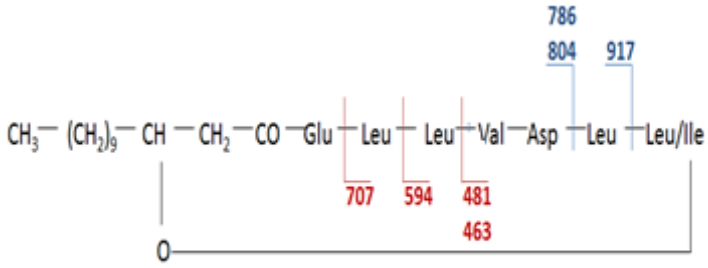

D
1512.9
51.9
1513.9⇐C18 Fengycin A [M+Na]+ / C16 Fengycin B [M+Na]+
C17 Fengycin A [M+Na]+/
C15 Fengycin B [M+Na]+
C17 Fengycin B
[M+Na]+
C19 Fengycin B
[M+Na]+
C16 Fengycin
A [M+Na]+
1499.9
1514.9
1527.9
1555.9
1498.9
1485.9
1542.9
1557.9
1526.9
1500.9
1569.9
1486.9
1515.9
1530.9
1543.9
1501.9
1600.0
1544.9
1573.0
1586.0
1614.0
1491.9
1525.9
1559.9
1566.9
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
1405
1457
1509
1561
1613
1665

Fig. 4

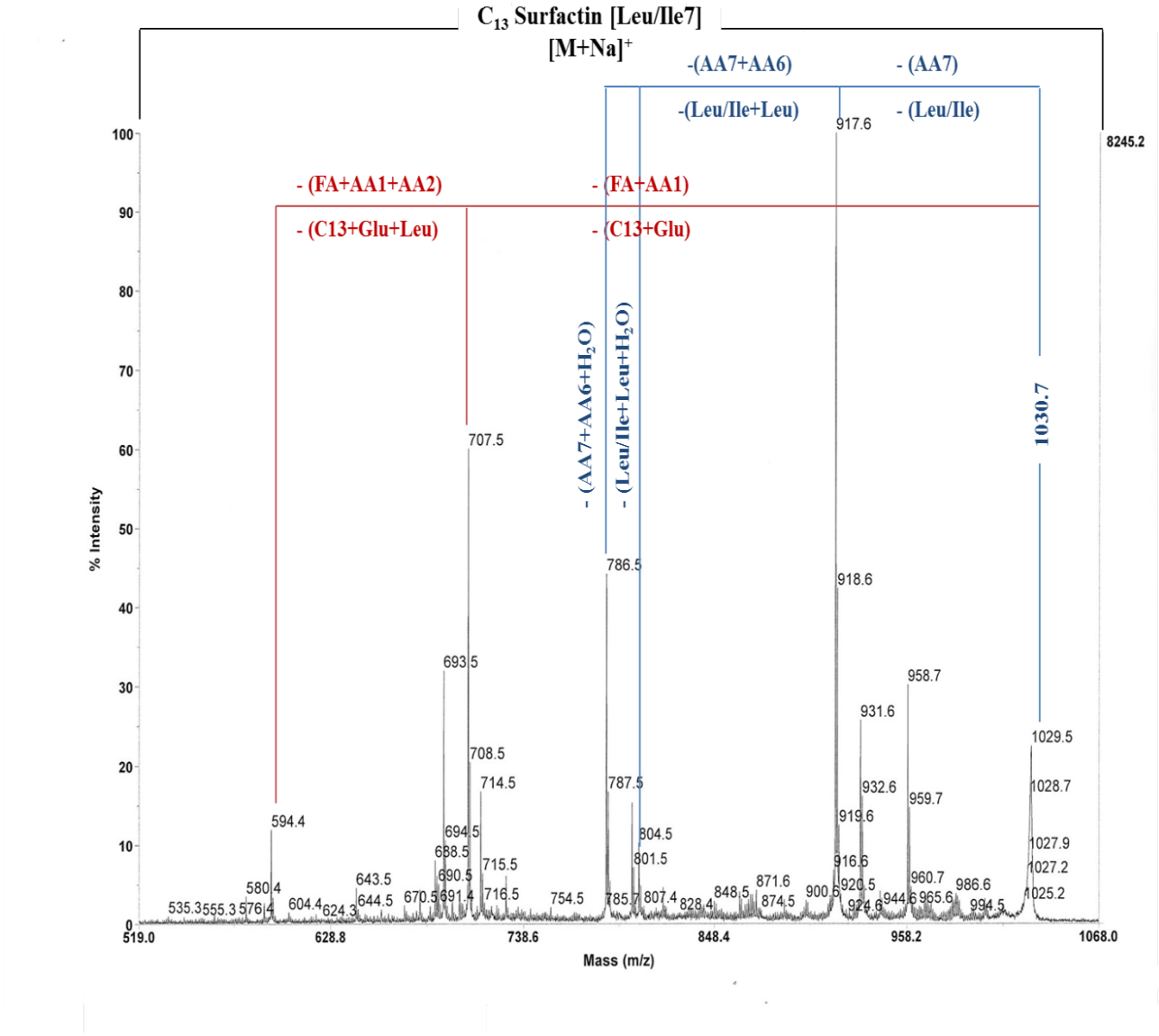

786
804
917
CH3 — (CH2)9 — CH — CH2 — CO — Glu — Leu — Leu — Val — Asp — Leu — Val
O

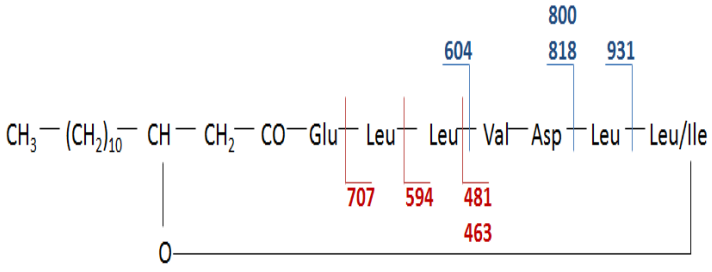
(a)


C13 Surfactin [Val7]
[M+Na]+
- (AA7+AA6)
- (Val+Leu)
- (AA7)
- (Val)
- (AA7+AA6+H2O)
- (Val+Leu+H2O)
1016.7
3139.1
903.6
% Intensity
Mass (m/z)
547.0
644.8
742.6
840.4
938.2
1036.0

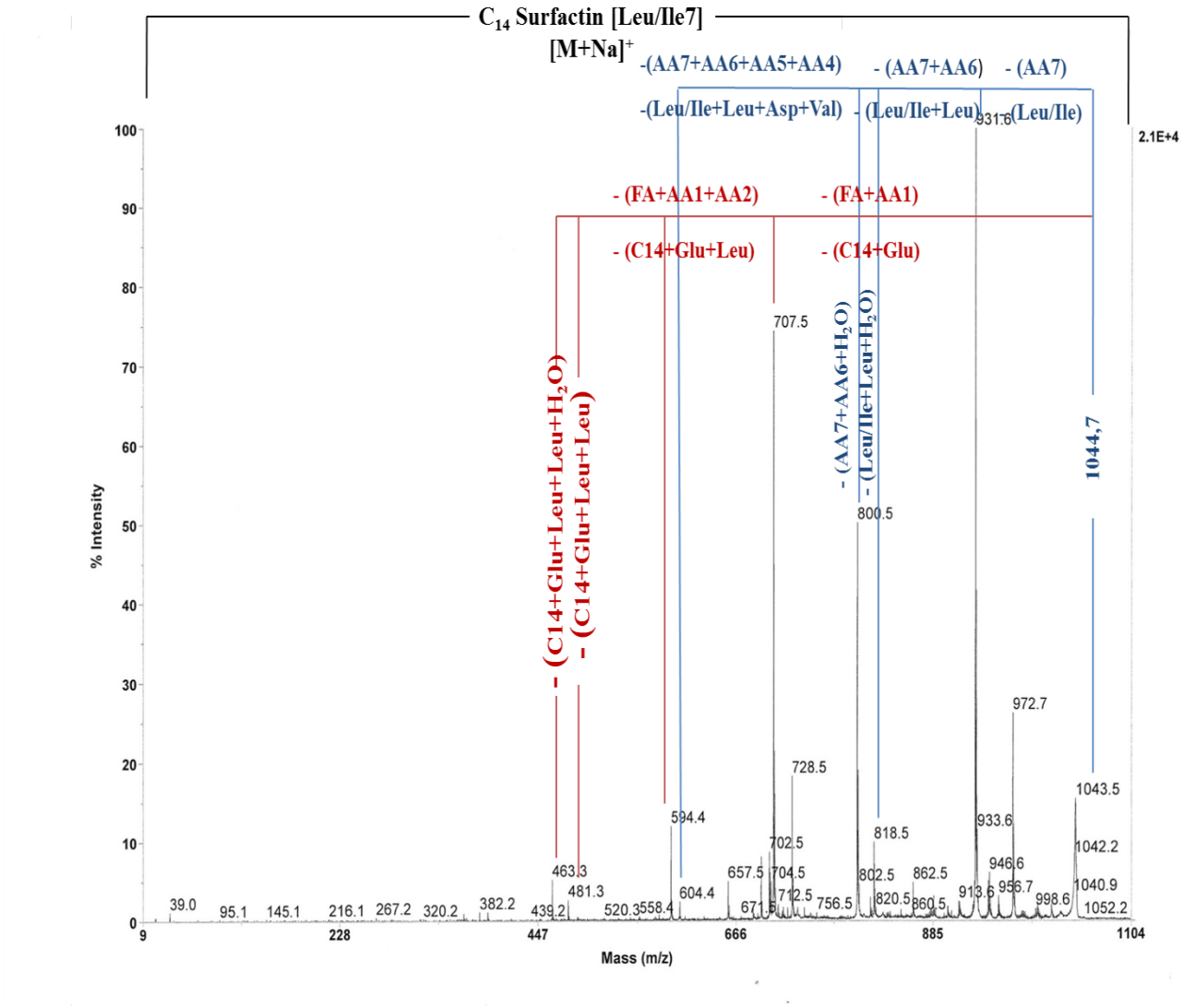

786
804
917
CH3 (CH2)9 CH CH2 CO Glu Leu Leu Val Asp Leu Leu/Ile
707
594
481
463
O

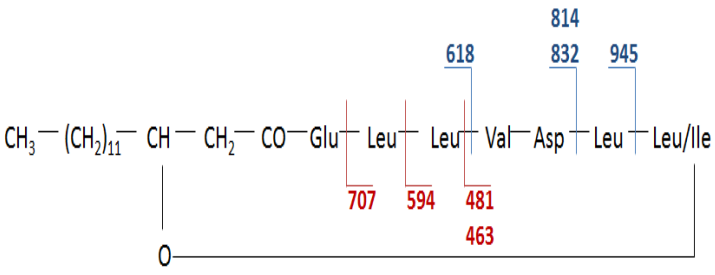
(b)


C13 Surfactin [Leu/Ile7]
[M+Na]+
-(AA7+AA6)
-(Leu/Ile+Leu)
- (AA7)
- (Leu/Ile)
- (FA+AA1+AA2)
- (C13+Glu+Leu)
- (FA+AA1)
- (C13+Glu)
- (AA7+AA6+H2O)
- (Leu/Ile+Leu+H2O)
1030.7
8245.2
% Intensity
Mass (m/z)
519.0
628.8
738.6
848.4
958.2
1068.0


800
604
818
931
CH3 — (CH2)10 — CH — CH2 — CO — Glu — Leu — Leu — Val — Asp — Leu — Leu/Ile
707
594
481
463
O

(c)


C14 Surfactin [Leu/Ile7]
[M+Na]+
-(AA7+AA6+AA5+AA4)
- (AA7+AA6)
- (AA7)
-(Leu/Ile+Leu+Asp+Val)
- (Leu/Ile+Leu)
(Leu/Ile)
931.6
2.1E+4
- (FA+AA1+AA2)
- (FA+AA1)
- (C14+Glu+Leu)
- (C14+Glu)
707.5
- (C14+Glu+Leu+Leu+H2O)
- (C14+Glu+Leu+Leu)
- (AA7+AA6+H2O)
- (Leu/Ile+Leu+Leu+H2O)
1044,7
800.5
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
9
228
447
666
885
1104
39.0
95.1
145.1
216.1
267.2
320.2
382.2
439.2
463.3
481.3
520.3
558.4
594.4
604.4
657.5
671.5
702.5
704.5
712.5
728.5
756.5
802.5
818.5
820.5
862.5
860.5
913.6
933.6
946.6
956.7
972.7
998.6
1040.9
1042.2
1043.5
1052.2


814
618
832
945
CH3 — (CH2)11 — CH — CH2 — CO — Glu — Leu — Leu — Val — Asp — Leu — Leu/Ile
707
594
481
463
O

(d)

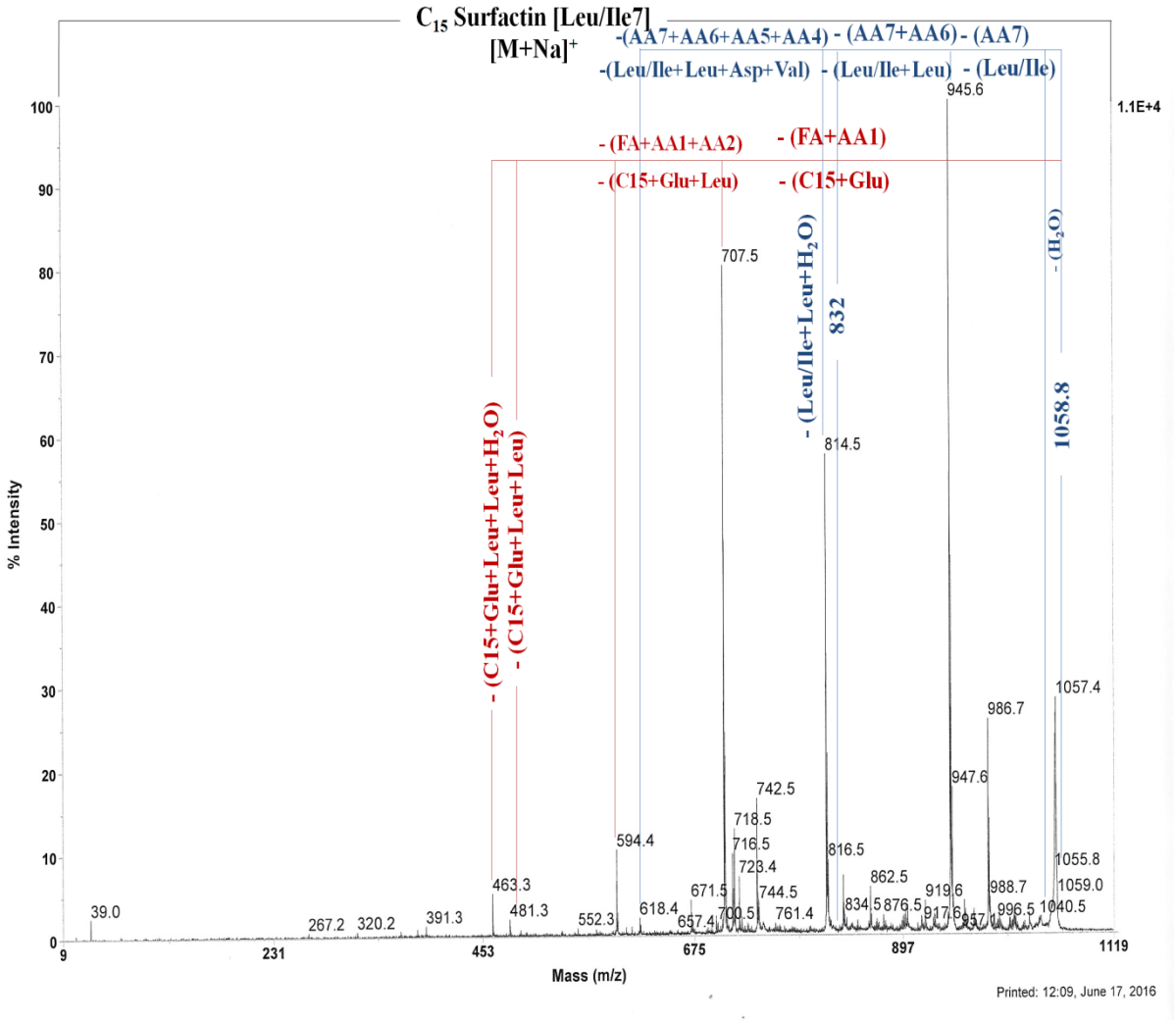
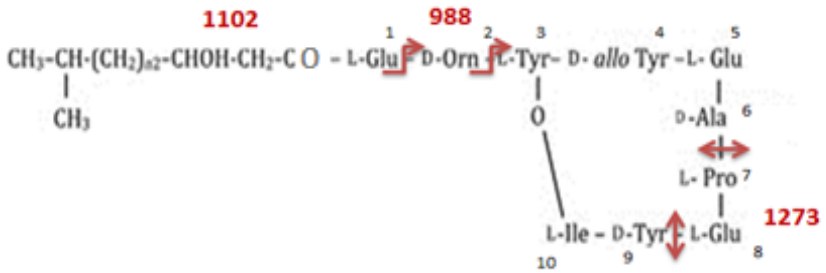
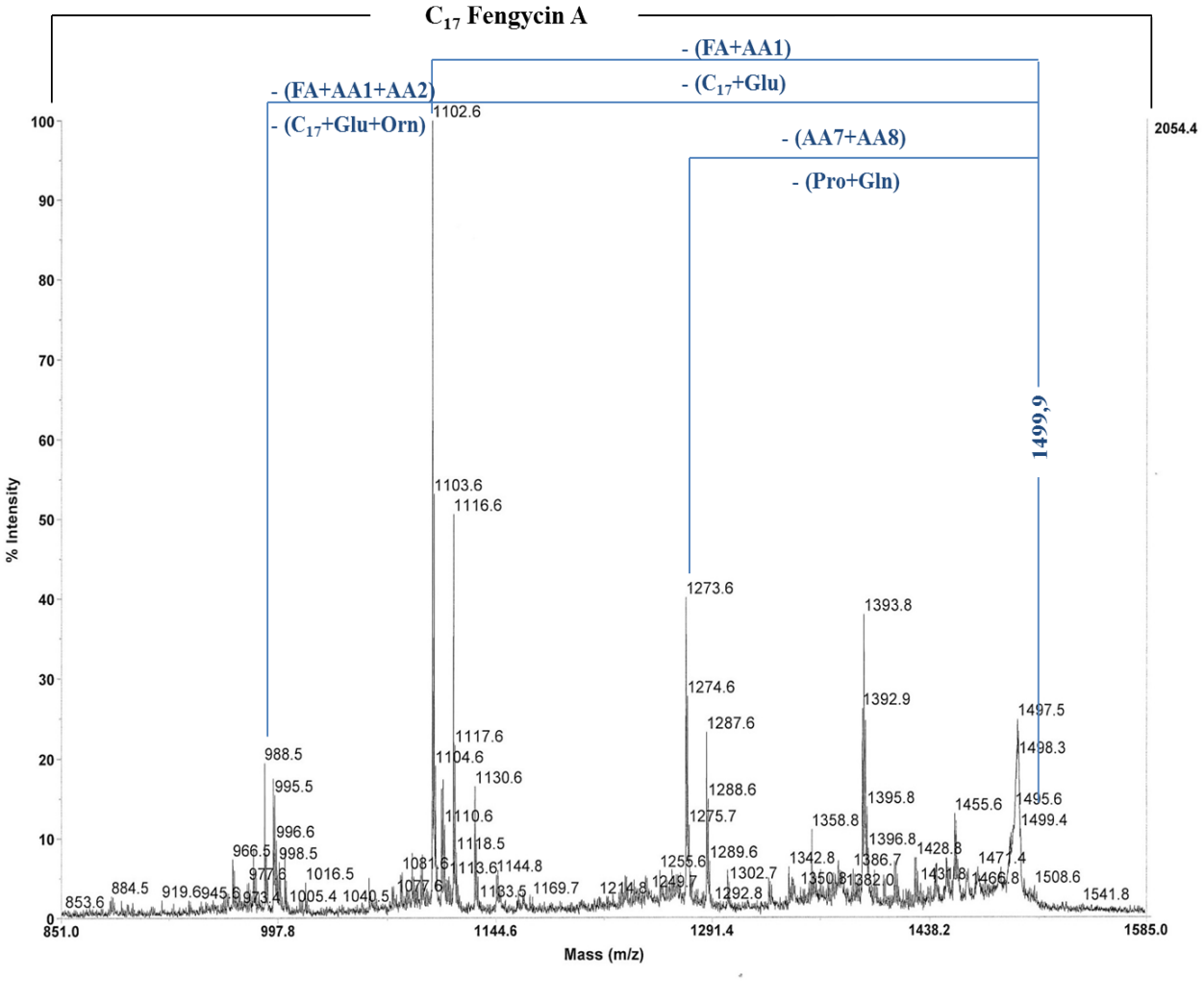
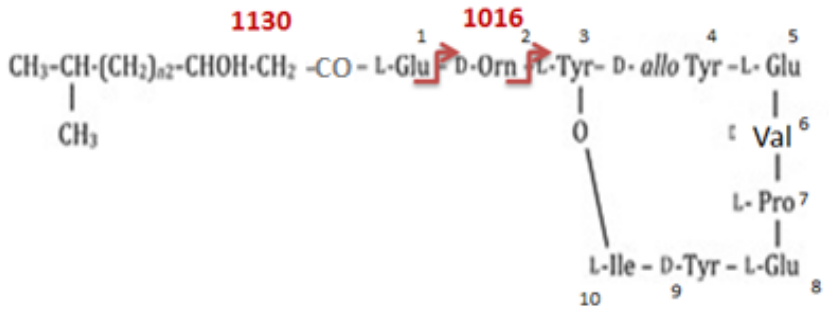

C15 Surfactin [Leu/Ile7]
[M+Na]+
-(AA7+AA6+AA5+AA4)
- (AA7+AA6)
- (AA7)
-(Leu/Ile+Leu+Asp+Val)
- (Leu/Ile+Leu)
- (Leu/Ile)
- (FA+AA1+AA2)
- (FA+AA1)
- (C15+Glu+Leu)
- (C15+Glu)
- (C15+Glu+Leu+Leu+H2O)
- (C15+Glu+Leu+Leu)
- (Leu/Ile+Leu+Leu+H2O)
- (H2O)
832
1058.8
945.6
707.5
814.5
1057.4
986.7
947.6
742.5
718.5
716.5
723.4
744.5
594.4
816.5
862.5
463.3
481.3
671.5
618.4
552.3
700.5
761.4
834.5
876.5
919.6
988.7
996.5
1040.5
1055.8
1059.0
39.0
267.2
320.2
391.3
% Intensity
Mass (m/z)
1.1E+4
Printed: 12:09, June 17, 2016

Fig. 5


1102
988
1273
CH3-CH-(CH2)a2-CHOH-CH2-C O - L-Glu - D-Orn - L-Tyr - D- allo Tyr - L- Glu
CH3
O
D-Ala
L- Pro
L-Ile - D-Tyr - L-Glu
1
2
3
4
5
6
7
8
9
10

(a)


C17 Fengycin A
- (FA+AA1)
- (C17+Glu)
- (FA+AA1+AA2)
- (C17+Glu+Orn)
- (AA7+AA8)
- (Pro+Gln)
1102.6
2054.4
1499,9
% Intensity
Mass (m/z)
851.0
997.8
1144.6
1291.4
1438.2
1585.0
100
90
80
70
60
50
40
30
20
10
0
853.6
884.5
919.6
945.6
966.5
973.4
977.6
988.5
995.5
996.6
998.5
1005.4
1016.5
1040.5
1077.6
1081.6
1103.6
1104.6
1110.6
1116.6
1117.6
1118.5
1130.6
1133.5
1144.8
1169.7
1211.8
1249.7
1255.6
1273.6
1274.6
1275.7
1287.6
1288.6
1289.6
1292.8
1302.7
1342.8
1350.8
1358.8
1382.0
1386.7
1392.9
1393.8
1395.8
1396.8
1428.8
1431.8
1455.6
1466.8
1471.4
1495.6
1497.5
1498.3
1499.4
1508.6
1541.8


1130
1016
CH3-CH-(CH2)n2-CHOH-CH2 -CO- L-Glu- D-Orn- L-Tyr- D- allo Tyr -L- Glu
CH3
Val
L- Pro
L-Ile - D-Tyr - L-Glu

(b)

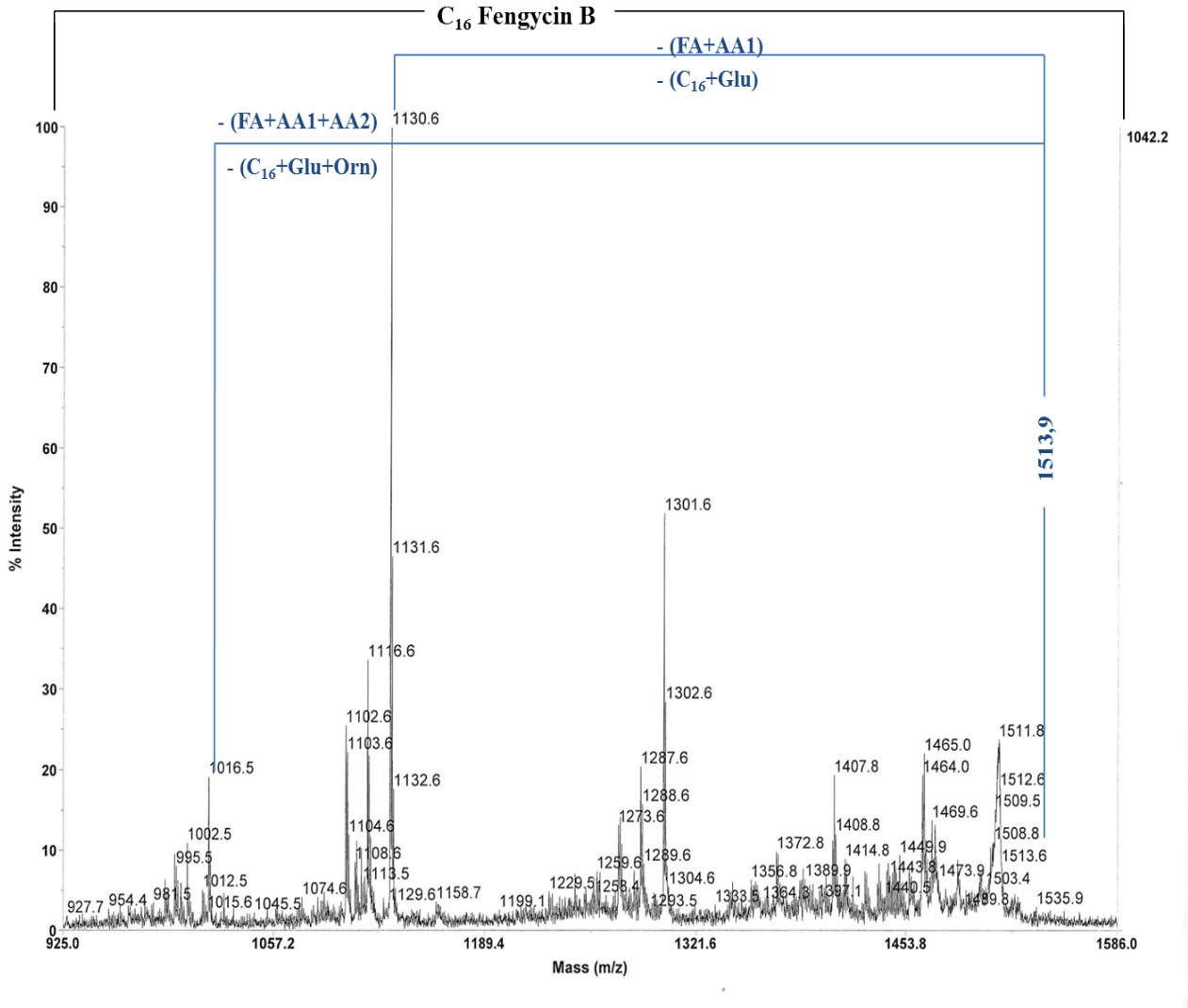

C16 Fengycin B
- (FA+AA1)
- (C16+Glu)
- (FA+AA1+AA2)
- (C16+Glu+Orn)
1130.6
1042.2
1513,9
% Intensity
Mass (m/z)
925.0
1057.2
1189.4
1321.6
1453.8
1586.0

Table 1. Total amino acids in the hydrolyzed crude lipopeptides DCS1

Amino acid	Molar ratio (%)
Glx	19.5
Asx	14.74
Tyr	8.45
Leu	8.33
Pro	7.49
Ser	6.29
Val	5.69
Ala	4.49
Gly	6.89
Lys	2.72
Phe	1.72
Arg	1.68
His	0.96

Table 2. Product ions from the fragmentation of surfactin precursor ions $[M + Na]^+$ at *m/z* 1030.7, 1044.7 and 1058.8

	m/z $[M + Na]^+$		
	1030.7	1044.7	1058.8
Peptidic series inside the C-terminal product ions			
$[M + Na - H_2O]^+$	-	-	1040.5
$[M + Na - Leu]^+$	917.6	931.6	945.6
$[M + Na - Leu\text{-}Leu]^+$	804.5	818.5	832.5
$[M + Na - Leu\text{-}Leu\text{-}H_2O]^+$	786.5	800.5	814.5
$[M + Na - Leu\text{-}Leu\text{-}Asp\text{-}Val]^+$	-	604.4	618.5
Fatty acid chain and the N-terminal product ions			
$[M + Na - FA\text{-}Glu]^+$	707.5	707.5	707.5
$[M + Na - FA\text{-}Glu\text{-}Leu]^+$	594.4	594.4	594.4
$[M + Na - FA\text{-}Glu\text{-}Leu\text{-}Leu]^+$	-	481.3	481.3
$[M + Na - FA\text{-}Glu\text{-}Leu\text{-}Leu\text{-} H_2O]^+$	-	463.3	463.3

Table 3. Different lipopeptides isoforms identified by MALDI-TOF mass spectrometry

Family	Mass peak (*m/z*)	Nature of the lipopeptide isoforms
Surfactin		
Surfactin	1016.7	C_{13} Surfactin [Val7] $[M+Na]^+$
	1030.7	C_{13} Surfactin [Leu7/Ile7] $[M+Na]^+$
	1044.7	C_{14} Surfactin [Leu7/Ile7] $[M+Na]^+$
	1058.8	C_{15} Surfactin [Leu7/Ile7] $[M+Na]^+$
Pumilacidin	1036.6	C_{15} Pumilacidin [Val7] $[M+H]^+$
	1050.6	C_{16} Pumilacidin [Val7] $[M+H]^+$
	1064.6	C_{17} Pumilacidin [Val7] $[M+H]^+$
	1078.7	C_{17} Pumilacidin [Ile7] $[M+H]^+$
Iturin		
Iturin/Mycosubtilin	1037.6	C_{12} Iturin A / C_{12} Mycosubtilin $[M+Na]^+$
	1051.6	C_{13} Iturin A / C_{13} Mycosubtilin $[M+Na]^+$
	1065.6	C_{14} Iturin A / C_{14} Mycosubtilin $[M+Na]^+$
	1079.7	C_{15} Iturin A / C_{15} Mycosubtilin $[M+Na]^+$
	1093.7	C_{16} Iturin A / C_{16} Mycosubtilin $[M+Na]^+$
Iturin C1	1092.7	C_{16} Iturin C1 $[M+Na]^+$
Bacillomycin D	1053.6	C_{14} Bacillomycin D $[M+Na]^+$
	1067.6	C_{15} Bacillomycin D $[M+Na]^+$
	1081.6	C_{16} Bacillomycin D $[M+Na]^+$
	1095.7	C_{17} Bacillomycin D $[M+Na]^+$
	1109.6	C_{18} Bacillomycin D $[M+Na]^+$
Fengycin		
Fengycin A	1449.9	C_{15} Fengycin A $[M+H]^+$
	1485.9	C_{16} Fengycin A $[M+Na]^+$
	1499.9	C_{17} Fengycin A $[M+Na]^+$
Fengycin B	1513.9	C_{16} Fengycin B $[M+Na]^+$
	1527.9	C_{17} Fengycin B $[M+Na]^+$
	1555.9	C_{19} Fengycin B $[M+Na]^+$

Highlights

- The crude biosurfactants DCS1 is composed of cyclic lipopeptides mixture with molecular weights between 1016 and 1556 Da.
- Mass spectrometry analysis of partially purified lipopeptides revealed that it contains various isoforms belonging to surfactin, iturin and fengycin families.
- MALDI-TOF MS^2 analysis was used to identify lipopeptides isoforms and to determine their chemical structure.