Isolation and characterization of kurstakin and surfactin isoforms produced by *Enterobacter cloacae* C3 strain

Nawel Jemil[a,*], Noomen Hmidet[a], Angeles Manresa[b], Francesc Rabanal[c] and Moncef Nasri[a]

[a]*Laboratoire de Génie Enzymatique et de Microbiologie, Université de Sfax, Ecole Nationale d'Ingénieurs de Sfax, B.P. 1173-3038 Sfax, Tunisia.*

[b]*Section of Microbiology, Department of Biology, Health and Environment, Faculty of Pharmacy, University of Barcelona, Joan XXIII s/n, 08028 Barcelona, Spain.*

[c]*Section of Organic Chemistry, Department of Inorganic and Organic Chemistry, Faculty of Chemistry, University of Barcelona, Martí i Franquès, 1, 08028 Barcelona, Spain*

* Corresponding author. Tel.: +216 50 498 815; fax: +216 74 275 595.
E-mail address: Nawel.1501@yahoo.com

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Abstract

In this work the extraction, structural analysis and identification as well as antimicrobial, anti-adhesive and anti-biofilm activities of lipopeptides produced by *Enterobacter cloacae* C3 strain were studied. A combination of chromatographic and spectroscopic techniques offers opportunities for a better characterization of the biosurfactant structure. Thin layer chromatography (TLC) and HPLC for amino acid composition determination are used. Efficient spectroscopic techniques have been utilized for investigations on the biochemical structure of biosurfactants, such as Fourier transform infrared (FT-IR) spectroscopy and mass spectrometry analysis. This is the first work describing the production of different isoforms belonging to kurstakin and surfactin families by *E. cloacae* strain. Three kurstakin homologues differing by the fatty acid chain length from C_{10} to C_{12} were detected. The spectrum of lipopeptides belonging to surfactin family contains various isoforms differing by the fatty acid chain length as well as the amino acids at positions four and seven. Lipopeptide C3 extract exhibited important antibacterial activity against Gram-positive and Gram-negative bacteria, antifungal activity and interesting anti-adhesive and disruptive properties against biofilm formation by human pathogenic bacterial strains: *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans*.

Keywords: *Enterobacter cloacae* C3; Kurstakin; Surfactin; Structure characterization; Anti-adhesive property.

Introduction

Biosurfactants are synthesized by a wide variety of microorganisms, mainly by bacteria and several yeasts.[1] Lipopeptides are among the most studied bioactive molecules, produced by multiple bacterial genera such as *Bacillus*,[2] *Paenibacillus*,[3] *Pontibacter*,[4] *Achromobacter*,[5] *Corynebacterium*,[6] *Pseudomonas*,[7] *Streptomyces*,[8] *Citrobacter* and *Enterobacter*.[9,10]

Lipopeptides are classified in various families and isoforms according to the peptide amino acid composition as well as the fatty acid chain length and the type of fatty acid binding. A common feature is the presence of an acyl chain bound to a cyclic peptide sequence; the peptide portion could be composed of either anionic or cationic residues with D or L configuration and might contain non-proteogenic or unusual amino acids. The peptide portion is non-ribosomally generated; the synthesis is directed by large multi-enzyme complex called Non-Ribosomal Peptide Synthetase (NRPS).[11] The most known lipopeptide families are: surfactin, iturin and fengycin-plipastatin. Surfactin and iturin lipopeptide compounds are cyclic lipoheptapeptides which contain a β-hydroxy and a β-amino fatty acid chain, respectively, as lipophilic moieties. Fengycin lipopeptides are cyclic lipodecapeptides with a β-hydroxy fatty acid chain. In addition to surfactin, iturin and fengycin families, kurstakin represents a new family of lipopeptides discovered in 2000 produced by *Bacillus thuringiensis* and it is considered as a biomarker of this species. Kurstakins were also detected in other species belonging to *Bacillus* genus such as *B. cereus*; they are lipoheptapeptides displaying antifungal activities.[12] The first isolated kurstakins did not contain a β-hydroxy fatty acid and were classified as linear molecules. It has been shown that they can be found in the form of partially cyclic compounds,[13] as well as in cyclic structures,[12] which places them in a class of non-cationic cyclic lipopeptides.[14] Cyclic lipopeptide biosurfactants like surfactin, iturin, bacillomycin, fengycin and kurstakin are

largely produced by species of the genus *Bacillus* which are Gram-positive bacteria exhibiting antimicrobial activity.[15,16] There are few studies describing the production of these lipopeptide families by Gram-negative bacteria.[9,10] Usually, Gram-negative bacteria of the genera *Pseudomonas*, *Klebsiella* and *Enterobacter* produce rhamnolipid and glycolipid biosurfactants.[17-20]

Different analytical techniques for chemical characterization of lipopeptides have been applied to elucidate their structure such as infrared spectroscopy (IR), amino acid analysis, high performance liquid chromatography (HPLC), capillary chromatography coupled to mass spectrometry (MS), gas chromatography (GC-MS) and UV/Vis spectroscopy,[21] nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS).[22] Furthermore, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) has proven to be very effective in the detection and identification of lipopeptides. Tandem mass spectrometry is a simple, fast, sensitive method and the appropriate technique to elucidate complex structures and mixtures on biological processes. Thousands of reports on applications of MS for microorganism characterization in research, clinical microbiology, food safety, environmental monitoring, and quality have been published.[23]

There is a high demand for new antimicrobial agents because of the increased resistance shown by pathogenic microorganisms against the existing antimicrobial drugs. Several natural lipopeptides produced by microorganisms have been developed as new therapeutic products and exploited for biomedical applications thanks to their antibacterial,[24,25] antifungal,[26] antiviral[27] and anti-adhesive properties[4,6,28] against several pathogenic microorganisms. In fact, some of the oldest available antibiotics in the market are cyclic antimicrobial peptides, such as polymyxins, gramicidin and bacitracin.[29,30]

The present work provides an insight into the search of new bioactive molecules from the Gram-negative bacteria *E. cloacae* C3. Chemical structure characterization and identification of different lipopeptide isoforms produced, as well as antimicrobial, anti-adhesive and anti-biofilm activities were carried out.

Experimental

Bacterial strain and biosurfactants production

The microorganism used in this study was isolated from soil at the area "Nakta" near the company "British gas", Sfax City, Tunisia, contaminated by natural-gas condensate, which comes from the gas of Miskar Asset field. It was identified as *Enterobacter cloacae* C3 strain based on the 16S rDNA gene sequence analysis.[31] It was inoculated into a 250 ml shake flask containing 25 ml Luria-Bertani broth medium and cultivated at 37 °C with shaking at 200 rpm for 18 h. A 3% (v/v) of inoculum [$OD_{600\ nm}$ = 6.7] was transferred into a 2 l shake flask containing 250 ml of Landy medium[32] and incubated in an orbital shaker at 30 °C and 150 rpm for 72 h.

Biosurfactants extraction

Biosurfactants recovery was performed as reported in our previous study.[33] Acid precipitated biosurfactant (1 g) was subjected to extraction with 45 ml tetrahydrofuran (THF) solvent four times and the mixture was stirred and centrifuged at 8000 rpm, for 15 min at 4 °C. The recuperated organic phases were combined and concentrated in a rotary vacuum evaporator (Büchi laborotechnik AG Postfach, Switzerland) at 40 °C.

Chemical characterization and identification of biosurfactants

FT-IR spectra of the crude dried biosurfactants

The functional groups and the chemical bonds present in the crude biosurfactants C3 were determined using Fourier transform infrared spectroscopy (FT-IR) in order to determine the chemical nature of biosurfactants. FT-IR analysis was performed by using Analect Instruments fx-6 160 FT-IR spectrometer at a wavenumber range 4000-400 cm^{-1}.

Thin layer chromatography (TLC) analysis

Biosurfactants extract was tested by TLC on silica gel plates 60 G (Macherel-Nagel, Düren, Germany) with mobile phase: chloroform/methanol/water (65:25:4). Staining was carried out with phosphomolybdic acid for the detection of lipids and o- Tolidine specific for peptide moiety, to detect the spots showing the presence of both fatty acid and peptide moieties.

Bradford assay for protein quantitation

The protein content of biosurfactants C3 was measured using Bradford Assay Kit through the microassay procedure as described in our previous study.[34]

Amino acid composition determination

The crude biosurfactants (4 mg) were hydrolyzed in 1 ml 6 M HCl at 110 °C overnight in a sealed tube. Aliquotes of AABA (L-α-Aminobutyric acid) and NLE (L-Norleucine) solutions were added as internal standards. Samples were evaporated to dryness and resuspended in water. The amino acids were then analyzed by HPLC with UV detection, using the Waters AccQTag pre-column derivatization method.[35]

Characterization of the lipopeptides by mass spectrometry (ESI and MALDI-TOF)

The molecular weight of the lipopeptide molecules was determined by positive/negative-ion modes 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/negative-ion modes, respectively, with nitrogen as the nebulizing and drying gas. Tandem mass spectrometry (4800 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^2 analysis.

Antimicrobial activities

The antimicrobial activity of C3 lipopeptides was estimated by agar well diffusion method against selected human pathogens. Antibacterial activity was tested against three Gram-positive bacteria: *Bacillus cereus* ATCC 11778, *Micrococcus luteus* ATCC 4698, *Staphylococcus aureus* ATCC 25923 and five Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Salmonella enterica* ATCC 27853, *Salmonella typhimurium* ATCC 19430 and *Enterobacterium* sp. Antifungal activity was tested against *Aspergillus niger*, *Aspergillus flavus* and *Fusarium oxysporum*.

The culture suspension (200 µl) of the tested microorganisms (10^6 colony-forming units cfu/ml) of bacteria cells (estimated by absorbance at 600 nm) and 10^8 spores/ml of fungal strains (measured by Malassez blade) were spread uniformly using sterile pipette on Luria-Bertani agar and malt extract agar media, respectively. Then, wells were made using a sterile well borer and were filled with 100 µl of lipopeptide sample (2 mg/ml concentration).

The zone of growth inhibition was measured in millimeters after incubation for 24 h at 37 °C for bacteria and for 72 h at 30 °C for fungal strains. All the results were represented as the average of three independent experiments with ≤ 5% deviation.

Anti-adhesion treatment with lipopeptide C3 extract

For surface pre-treatment, the wells of a sterile polystyrene microtiter plate (Costar; Corning Incorporated, Corning, NY, USA) were filled with 200 µl of C3 lipopeptide extract at different concentrations ranging from 0.008 to 1 mg/ml, dissolved in PBS composed of (g/l): NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.44; KH_2PO_4, 0.24 (pH 7.2). Microtiter plates were incubated for 6 h at room temperature (25 °C) and then washed twice with PBS.

For biofilm formation, *Klebsiella pneumoniae* ATCC 1388, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, *Bacillus cereus* ATCC 11778 and *Candida albicans* ATCC 10231 were cultured overnight in Luria Bertani medium (LB). Cultures were diluted 1/100 in the medium proposed by O´Toole[36] (g/l): glucose, 2; casamino acids, 5; KH_2PO_4, 3; K_2HPO_4, 7; $(NH_4)_2SO_4$, 2, $MgSO_4\,7H_2O$, 0.12. Then, 200 µl of each dilution were added to the microtiter plate wells and plates were incubated for 20 h at 37 °C. Cultures were discarded and wells were washed three times with distilled water to remove non-adherent cells, fixed for 15 min with methanol and stained with 125 µl crystal violet (0.1%, w/v) for 20 min, then washed with water and drying. For quantifying the microbial adhesion, 200 µl of acetic acid in water (33%, v/v) were added and the absorbance was determined at 595 nm. Percentages of microbial adhesion inhibition were calculated using the following formula:

$$\text{Microbial adhesion inhibition} = [1 - (Ac/A0)] \times 100$$

where A_c represents the absorbance of the well with lipopeptides at concentration c and A_0 represents the absorbance of the positive control wells (in absence of lipopeptides). Negative control wells contained only lipopeptides dissolved in PBS. Assays were carried out three times with ≤ 5% deviation.

Mature biofilm treatment with lipopeptide C3 extract

The wells of a sterile polystyrene microtiter plate were loaded with 200 µl of bacterial suspension prepared as mentioned above, then the plates were incubated for 20 h at 37 °C. After incubation, the unattached microbial cells were removed by washing the wells three times with distilled water. Then, 200 µl of C3 lipopeptides at different concentrations ranging from 0.008 to 1 mg/ml, were added to each well and the plates were incubated for 6 h at room temperature (25 °C). The quantification was carried out as in the pre-treatment. All the results were represented as the average of three independent experiments with ≤ 5% deviation.

Methods of analysis

All data presented are the average of at least three measurements which deviated by not more than 5%.

Results and discussion

Chemical structure characterization of biosurfactants

Preliminary chemical characterization

The IR spectrum of the crude biosurfactants from *E. cloacae* C3 strain showed several strong bands (Figure 1). The peak with the highest absorbance in the spectrum at 1655 cm^{-1}, results from the stretching mode of the carbonyl group (C=O) of the amide bond (-CONH-), also there is a small contribution from carbonyls of the ester bond and carboxyl side chains of

some amino acids such as Glu, Gln, Asp and Asn indicating the presence of peptide groups in the molecule.[37] Adjacent to this peak, there is another high intensity peak at 1540 cm^{-1} resulting from the deformation mode of N–H bonds.[38] The absorbance peaks at 2959, 2928 and 2850 cm^{-1} indicate the presence of C–H bonds of the alkyl chains. Another peak at 1405 cm^{-1} corresponds to C–H bending vibrations, it is common in compounds with alkyl chains. The ester carbonyl group is detected from the absorbance peaks at 1057, 1254 and 1104 cm^{-1}. The strong peak at 3300 cm^{-1} can be attributed to the presence of carboxyl side chains of glutamic/aspartic acids (O-H stretching) and –NH bonds of the amide group which overlaps the stretching in the same region. The observed peaks are similar to those reported by Das et al.[39] and Jemil et al.[28] for lipopeptide biosurfactants. The lipopeptide biosurfactant, surfactin (Sigma) also yielded a similar IR absorption pattern and absorbed approximately at the same wavenumber positions.[39]

The characterization of biosurfactant C3 extract by TLC analysis showed many spots at different levels of migration after spraying with phosphomolybdic acid reagent, yellow color was revealed after treatment with o- Tolidine which correspond to the presence of peptide moieties. The appearance of many spots at different levels of migration suggest the presence of lipopeptide molecules with different polarities.

Amino acid composition determination

The amino acid content of the crude lipopeptides synthesized by *E. cloacae* C3 strain was determined and results are presented in Table 1. 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, respectively. The sample of the crude lipopeptides C3 has a 36% of peptide content. Amino acids with high molar ratio are Leu, Glx and Asx with percentages of 13.54%, 12.47% and 11.39%, respectively. The amino acid Leu is present with three or four

residues in surfactin lipopeptide and with four or five residues in pumilacidin lipopeptide. The amino acids Glu or Gln are present with one or three residues in different lipopeptide isoforms belonging to different families. Asp is in the composition of surfactin and pumilacidin with one residue.

Amino acids Gly and Ala are present in lipopeptides with percentages of about 9.0% and 8.0%, respectively. These two amino acids are in the composition of lipopeptides belonging to kurstakin family with one residue. Val and Thr have molar ratios of 6.6% and 6.0%, respectively, in lipopeptides C3. The amino acid Val is with one or two residues in surfactin lipopeptide and Thr is with one residue in kurstakin molecules. Also, Ile and Ser have molar ratios of 5.5% and 5.3%, respectively. The amino acid Ile is in the composition of lipopeptides belonging to surfactin family with one residue at most and Ser is in the composition of kurstakin lipopeptide with one residue. While, the amino acid His with 2.0% molar ratio, is present with one residue at position 5 in the peptide moiety of kurstakin isoforms.

Detection of lipopeptides by mass spectrometry analysis

Mass spectrometry analysis of lipopeptide C3 extract reported in Figure 2 shows the presence of two well-resolved clusters of peaks, the first at *m/z* values between 887.5 and 915.6 (Figure 2A) and the second within the mass range 1044.7 and 1100.7 Da (Figure 2B). By comparing the mass (*m/z*) with the mass values reported for others identified lipopeptides,[12,34,40] we can conclude that the first group of peaks (887.5 - 915.6 Da) corresponds to kurstakin lipopeptide and the second (1044.7 - 1100.7 Da) corresponds to lipopeptides belonging to surfactin family.

Identification of kurstakin isoforms

Figure 2A shows different peaks corresponding to kurstakin lipopeptide isoforms. The precursor ion at *m/z* 887.5 corresponds to a sodium ion adduct of cyclic kurstakin lipopeptide with a fatty acid chain of 10 carbon atoms. Other lipopeptide homologues detected at *m/z* 901.6 and 915.6 could be attributed to C_{11} and C_{12} kurstakin $[M + Na]^+$, respectively. Each adduct yields an isotopic distribution of 3 peaks differing by 1 mass unit.

Kurstakin lipopeptide synthesized by *E. cloacae* C3 strain consist of cyclic lipoheptapeptides with fatty acid chains from C_{10} to C_{12} differing by (*m/z* of CH_2 = 14 Da) and a peptidic sequence composed of L Thr - Gly - L Ala - L Ser - L His - L Gln - L Gln with the presence of an amide bond between the fatty acid chain and the first threonine residue and the presence of a lactone linkage between the serine at position 4 and the C terminus of glutamine at position 7. Abderrahmani et al.[41] detected the presence of the three kurstakin isoforms C_{11}, C_{12} and C_{13} in six *B. thuringiensis* strains. According to Béchet et al.[12], kurstakins were typically identified by the molecular ions at *m/z* 889, 905, 917 and 933.

Identification and characterization of surfactin and pumilacidin lipopeptides by tandem mass spectrometry

The precursor ions at *m/z* 1044.7, 1058.7, 1072.7, 1086.7 and 1100.7 (Figure 2B) were assigned as the sodium ion adducts of homologous surfactin lipopeptides with 1021.7, 1035.7, 1049.7, 1063.7 and 1077.7 Da mass, respectively. The structure characterization of surfactin lipopeptides was elucidated by MS^2 fragment analysis. The tandem mass spectrometry analysis was used to carry out the fragmentation of lipopeptides in order to obtain more precise information on their chemical structure. However, there is more ambiguity in the fragmentation of the parent ions detected by LC/MSD-TOF analysis and we

obtained two different fragmentation models for each peak corresponding to two different molecules (Figures 3 and 4).

The fragmentation patterns of the peak 1044.7 were illustrated in Figure 3a. The first fragmentation model resulted in the appearance of product ions from the C-terminal tail of the aliphatic peptide moiety at *m/z* 945.6, 832.6 and 717.5 corresponding respectively, to the losses of Val (−99 Da), Val-Leu (−212 Da) and Val-Leu-Asp (−327 Da) from the parent ion *m*/*z* 1044.7. Therefore, the amino acid at position 7 is Val. An other product ion resulted from the fragmentation on the side of the fatty acid chain and the N-terminal tail, *m*/*z* observed was 693.4 corresponding to the losses of C_{15} *β*-hydroxy fatty acid chain-Glu (−351 Da) from the precursor ion *m*/*z* 1044.7. The obtained results indicated that the peak at *m/z* 1044.7 may corresponds to surfactin, a cyclic lipopeptide with a fatty acid chain of 15 carbon atoms and Val residue at position 7. The second fragmentation pattern resulted in the appearance of product ions from the C-terminal tail of the aliphatic peptide moiety at *m/z* 931.6, 818.5 and 800.5 corresponding respectively, to the losses of Leu/Ile (−113 Da), Leu/Ile-Leu (−226 Da) and Leu/Ile-Leu-H_2O (−244 Da) from the parent ion *m*/*z* 1044.7. Therefore, the amino acid at position 7 is Leu/Ile. Other product ions resulted from the fragmentation on the side of the fatty acid chain and the N-terminal tail, *m*/*z* observed were 594.4 and 463.3 corresponding respectively, to the losses of C_{14} *β*-hydroxy fatty acid chain-Glu-Leu (−450 Da) and C_{14} *β*-hydroxy fatty acid chain-Glu-Leu-Leu-H_2O (−581 Da) from the precursor ion *m*/*z* 1044.7. The obtained results indicated that the peak at *m/z* 1044.7 may also corresponds to surfactin, a cyclic lipopeptide with a fatty acid chain of 14 carbon atoms and Leu/Ile residue at position 7. Thus, we can conclude that this lipopeptide may corresponds to C_{14} surfactin [Leu/Ile7] and C_{15} surfactin [Val7].

The same fragmentation sites were observed for the parent ions *m*/*z* 1058.7, 1072.7, 1086.7 and 1100.7 (Figures 3 and 4). Results showed that these lipopeptides correspond to

C_{16} surfactin [Val7] and C_{15} surfactin [Leu/Ile7], C_{16} pumilacidin [Val7] and C_{15} pumilacidin [Leu/Ile7], C_{17} pumilacidin [Val7] and C_{16} pumilacidin [Leu/Ile7], and C_{17} pumilacidin [Leu/Ile7], respectively.

The precusor ions at *m/z* values of 1044.7 and 1058.7 correspond to surfactin isoforms differing by the acid chain length (*m/z* of CH_2 = 14 Da). Also, the difference between the sodiated molecules $[M + Na]^+$ at *m/z* 1058.7 and 1072.7 is 14 Da, these two lipopeptides have the same acid chain length but differ by the amino acid at position 4, which is valine for surfactin and leucine for pumilacidin lipopeptide. Based on the fragmentation results, the precursor ions at *m/z* values of 1072.7, 1086.7 and 1100.7 were assigned as the sodium ion adducts of pumilacidin isoforms differing by the acid chain length. According to Pabel et al.[42], the mass peak 1086.9 was assigned to sodiated C_{17} pumilacidin.

Our results of fragmentation of the parent ions at *m/z* 1044.7 and 1058.7 resulting in the sodiated lipopeptides C_{14} surfactin [Leu/Ile7] and C_{15} surfactin [Leu/Ile7], respectively, are in accordance with those demonstrated by Pecci et al.[43], Jemil et al.[34] and Dimkić et al.[44], who characterized lipopeptides produced by *B. licheniformis* V9T14, *B. methylotrophicus* DCS1 and *Bacillus* spp. strains, respectively. Also, according to You et al.[10], the fragmentation of the parent ion at *m/z* 1058.6 was recognized to be sodium adducts of C_{15} surfactin. Plaza et al.[45] reported that the sodiated molecules $[M + Na]^+$ *m/z* 1044, 1058 and 1072 correspond to C_{14}, C_{15} and C_{16} surfactin homologues, respectively, obtained from lipopeptides produced by *B. subtilis* KP7 strain. According to Savadogo et al.[46], two strains *B. subtilis* S6 and *B. licheniformis* S12 produce biomolecules with *m/z* related to $[M + Na]^+$ forms of surfactin C_{14} (*m/z* 1044) and $[M + Na]^+$ forms of surfactin C_{15} (*m/z* 1058). In another study, Chen et al.[40] reported that the sodium peaks at *m/z* 1044 and 1058 are the characteristic peaks of surfactin molecular weight produced by *B. licheniformis* MB01.

This is the first work describing kurstakin, surfactin and pumilacidin lipopeptide mixture production from *E. cloacae* strain. In fact, the study of You et al.[10] describes the production of surfactin homologues from *Enterobacter* sp. N18 strain. Mandal et al.[9] reported that the comprehensive mass spectral (MALDI-TOF-MS and GC-MS) analysis of HPLC purified antimicrobial lipopeptides obtained from *E. cloacae* subsp. *dissolven*s S-11 strain revealed the occurrence of C_{17} fengycin B'2, C_{14} iturin and C_{15} kurstakin. Whereas, the antimicrobial lipopeptide obtained from *E. homaechei* S-5, *E. mori* S-9, *Enterobacter* sp. S-4, *E. cloacae* subsp. *dissolven*s S-10 and S-12 is C_{15} kurstakin.

Antimicrobial activities

The antimicrobial activities of lipopeptide mixture produced by *E. cloacae* C3 strain were tested against different Gram-positive, Gram-negative bacteria and fungi strains. The results showed that lipopeptides C3 exhibited interesting antibacterial and antifungal activities (Table 3). *K. pneumoniae* is the most sensitive strain toward antibacterial activity of lipopeptides C3 with a maximum zone diameter inhibition of 35 mm, while the lowest inhibition activity was observed against *S. aureus* with a zone diameter inhibition of 14 mm. However, lipopeptides C3 did not exhibit antibacterial activity against *B. cereus*, *E. coli*, *S. enterica* and *Enterobacterium* sp. at 2 mg/ml concentration. The inhibitory activity was more effective against Gram-negative bacteria compared to Gram-positive bacteria with inhibition zones diameters in the range of 28-35 mm and 14-18 mm, respectively. Our results are in contrast with those of Ben Ayed et al.[25] who reported that Gram-positive bacteria are more sensitive to the inhibitory activity of lipopeptides produced by *B. mojavensis* A21 strain, compared to Gram-negative bacteria.

Surfactin lipopeptides are the first and the most well-known member by their antimicrobial activities. According to Iyer and Sandhya[47], the maximum inhibition activity

of the crude surfactin sample (1.5 mg/ml) was observed against *Salmonella paratyphi* A, *Staphylococcus* sp. and *E. coli* with a zone diameter inhibition of 6 mm. Also, Sivapathasekaran et al.[48] showed the antibacterial activity of HPLC purified fractions containing surfactin. Mandal et al.[9] reported that lipopeptides belonging to kurstakin, iturin and fengycin families produced by *Citrobacter* S-3 and *Enterobacter* S-11 strains, have an unusual broad sprectrum antibacterial activity. Lipopeptides which differ in their composition, follow the same mechanisms such as involving pore formation on bacterial membrane[49] or by other non-specific interactions with the membrane[50] as a result of their antimicrobial activity. Sotirova et al.[51] reported that biosurfactants act disturbing the cytoplasmic membrane, as they have an amphipathic nature that allows its interaction with phospholipids, altering permeability with consequent cell damage.

Lipopeptides produced by *E. cloacae* C3 strain showed an interesting antifungal activity against *A. niger* and *F. oxysporum*, but without inhibitory effect against *A. flavus*. Some other results have mentioned the antifungal activity of surfactins[52-55] which would participate to the preservation of the products against molds. Similar results are reported by Jadhav et al.[20] who showed that biosurfactants produced by *Enterobacter* sp. MS16 strain exhibited a potential antifungal activity and inhibit fungal spores germination. The antimicrobial activities of lipopeptides C3 may be related to a synergistic effect of both surfactins and kurstakins. The antimicrobial properties of biosurfactants have been widely reported. However, the biosurfactants with antimicrobial properties reported till date are produced mostly by the terrestrial origin microorganisms as a part of defence mechanism to survive in complex environments.[56]

Anti-adhesive activity

Adhesion to surfaces and biofilm formation is a surviving strategy used by microorganisms in many environments, protecting them from dehydration, biocides and extreme conditions.[57] Biosurfactants have a great influence on the process of biofilm formation due to their strong anti-adhesive properties.[58] Lipopeptides produced by *E. cloacae* C3 strain exhibited a potential antiadhesive activity against all microorganisms tested even at very low concentrations (Figure 5a). Inhibition of biofilm formation increased with increasing lipopeptide concentration and the rate of inhibition remains nearly constant above a concentration of 0.5 mg/ml with all microorganisms tested. A very high antiadhesive capacity was observed against *B. cereus*, *S. typhimurium* and *C. albicans* with inhibition percentages of 96.7%, 92% and 89.3%, respectively. A high inhibition percentage was also obtained against *K. pneumoniae* and *S. aureus* with 70.4% and 60.80% inhibition, respectively. Lipopeptides C3 were very effective against *C. albicans*, they reached nearly the maximum of biofilm formation inhibition (85%), at a very low concentration of about 0.03 mg/ml. Araujo et al.[59] reported that surfactin significantly reduced adhesion of *Listeria monocytogenes* ATCC 19112 on polystyrene surfaces with 54% inhibition, when used at a concentration of 0.50% (w/v).

Lipopeptides produced by *E. cloacae* C3 strain are highly effectives, having a very low calculated effective dose (ED_{50} with 50% adhesion inhibition) with all microorganisms tested: 5, 100, 130, 346 and 453 µg/ml for *C. albicans*, *B. cereus*, *S. typhimurium*, *K. pneumoniae* and *S. aureus*, respectively. The prior adsorption of lipopeptides to solid surfaces might constitute a new and effective strategy to reduce microbial adhesion and preventing colonization by pathogenic microorganisms, not only in the biomedical field, but also in the food industry.[60,61] This effect could be related to biosurfactants influence on the reduction of bacterial cell hydrophobic properties or on the repulsion between bacteria and abiotic

surfaces.[62] According to Araujo et al.[59], biofilm formation is inhibited by the conditioning of polystyrene and stainless steel 304 with rhamnolipids and surfactin biosurfactants, transforming the surfaces hydrophilic or less hydrophobic compared to the control. The decrease in surface hydrophobicity as a result of conditioning by biosurfactants entails a decrease in hydrophobic interactions with cell wall of microorganisms and as a result, adhesion/biofilm formation is reduced.

Disruptive activity on pre-formed biofilm

In order to assess the potential of lipopeptides to remove biofilms, the cultures of the pathogens were treated with the mixture of lipopeptides C3 at different concentrations. They disrupted the biofilms of all tested microorganisms at different levels. As shown in Figure 5b, disruptive effect is dose dependent and percentages remain nearly constants above lipopeptide concentration of 0.5 mg/ml. The greatest biofilm disruption activity produced by lipopeptides C3 was observed against *C. albicans* with a percentage of 89.7%, followed by 87% against *S. typhimurium*, 77.7% against *S. aureus*, 71.3% against *K. pneumoniae* and 70.3% against *B. cereus*. Our findings are in disagree with those of Coronel-león et al.[63] who reported that lichenysin produced by *B. licheniformis* AL1.1 is not very potential in the removal of biofilm formed by *C. albicans* ATCC 10231 (37.97%) at a concentration of 4 mg/ml. The effectiveness of lipopeptides in removing pre-formed biofilms using different microorganisms is similar to that in preventing the formation of these biofilms. Results obtained are in accordance with our findings in a previous study showing that lipopeptides belonging to surfactin, iturin and fengycin families produced by *B. methylotrophicus* DCS1 strain are effectives in pre-treatment as well as in post-treatment of biofilm formation.[28]

The effective dose (ED_{50}) is very low in post-treatment with all microorganisms tested: 8, 44.28, 156, 260 and 273 µg/ml for *S. typhimurium*, *K. pneumoniae*, *B. cereus*, *C. albicans*

and *S. aureus*, respectively. Biosurfactants can adsorb at the interface between the attached biofilm-forming bacteria and the solid surface by orienting polar and nonpolar groups. This interaction between biosurfactants and the surface alters the surface hydrophobicity, thereby interfering with microbial adhesion and desorption processes.[64,65] The results suggest that lipopeptides produced by *E. cloacae* C3 strain are potential against all microorganisms tested.

Conclusion

In this study, different cyclic lipopeptides belonging to kurstakin and surfactin families were detected in *E. cloacae* C3 strain and their structures were elucidated through tandem mass spectrometry. Twelve lipopeptide variants belonging to the two different families were identified; lipopeptide isoforms differ by the fatty acid chain length as well as the amino acid composition of the peptide cycle. These lipopeptides exhibited an important antimicrobial activity mainly against *K. pneumoniae*. In addition, they displayed an excellent anti-adhesive and disruptive properties against biofilm formation by a variety of bacteria. In conclusion, *E. cloacae* C3 strain is a good biocontrol and therapeutic agent for use in combating many diseases and infections thanks to the antimicrobial and anti-adhesive properties of lipopeptides produced.

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Table 1. Total amino acids in the hydrolyzed crude lipopeptides C3

Amino acid	Molar ratio (%)
Leu	13.54
Asx	12.47
Glx	11.39
Gly	8.91
Ala	8.11
Val	6.64
Thr	6.03
Ile	5.50
Ser	5.29
Pro	3.22
Tyr	3.02
His	2.01
Phe	3.42
Arg	3.15

Table 2. Different lipopeptide isoforms identified by mass spectrometry

Family	$[M + Na]^+$ ion	Nature of lipopeptide isoforms
Kurstakin	887.5	C_{10} Kurstakin
	901.6	C_{11} Kurstakin
	915.6	C_{12} Kurstakin
Surfactin	1044.6	C_{14} Surfactin [Leu7/Ile7] and C_{15} Surfactin [Val7]
	1058.6	C_{15} Surfactin [Leu7/Ile7] and C_{16} Surfactin [Val7]
	1072.6	C_{15} Pumilacidin [Leu7/Ile7] and C_{16} Pumilacidin [Val7]
	1086.6	C_{16} Pumilacidin [Leu7/Ile7] and C_{17} Pumilacidin [Val7]
	1100.7	C_{17} Pumilacidin [Leu7/Ile7]

Table 3. Antimicrobial activity spectrum of C3 lipopeptides (2 mg/ml)

Indicator organisms	**Inhibition zone diameter (mm)**
Gram (+)	
S. aureus (ATCC 25923)	14 ± 1.4
B. cereus (ATCC 11778)	-
M. luteus (ATCC 4698)	18 ± 0
Gram (-)	
K. pneumoniae (ATCC 13883)	35 ± 0,7
E. coli (ATCC 25922)	-
S. typhimurium (ATCC 19430)	28 ± 0,7
S. enterica (ATCC 27853)	-
Enterobacterium sp.	-
Fungi	
A. niger	+++
A. flavus	-
F. oxysporum	++

Determinations were performed in triplicate and data correspond to mean values ± standard deviations.

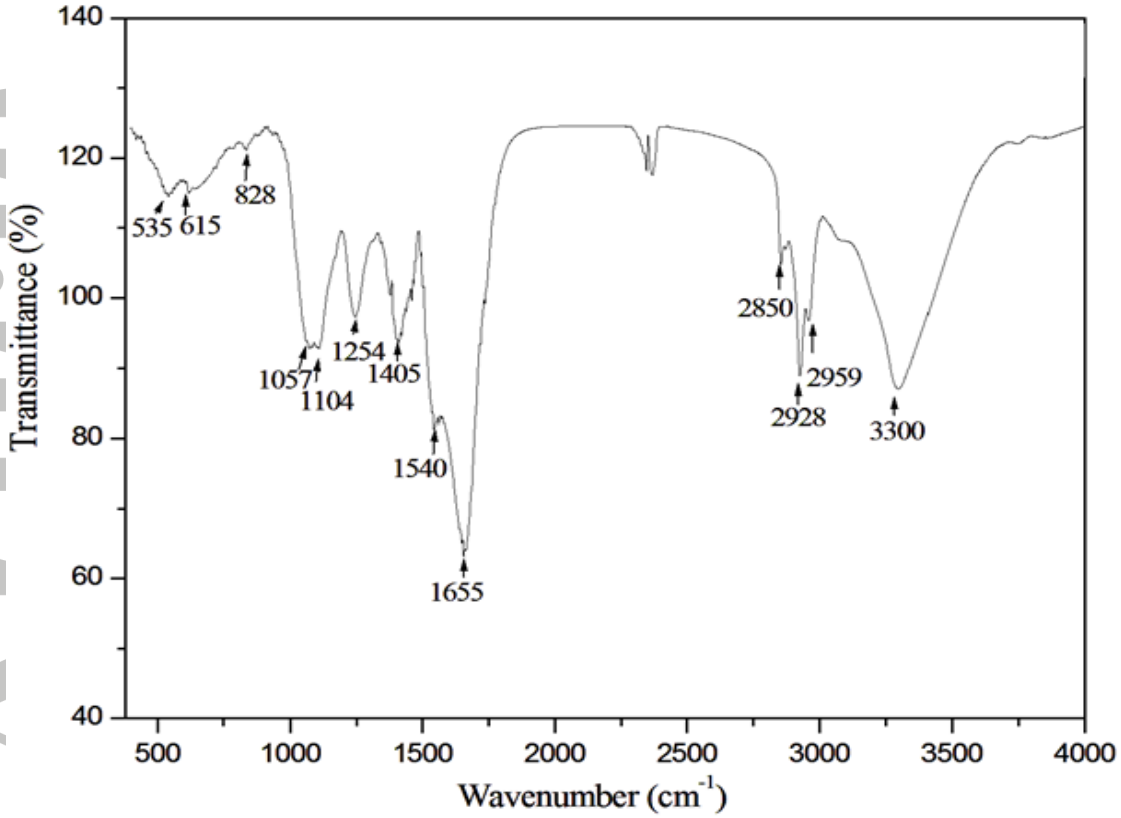
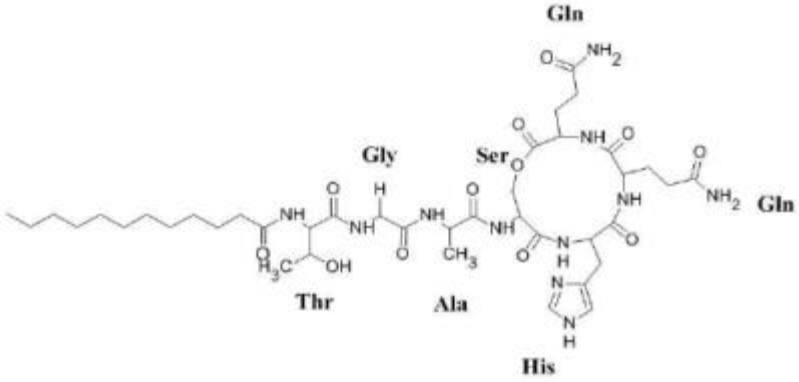
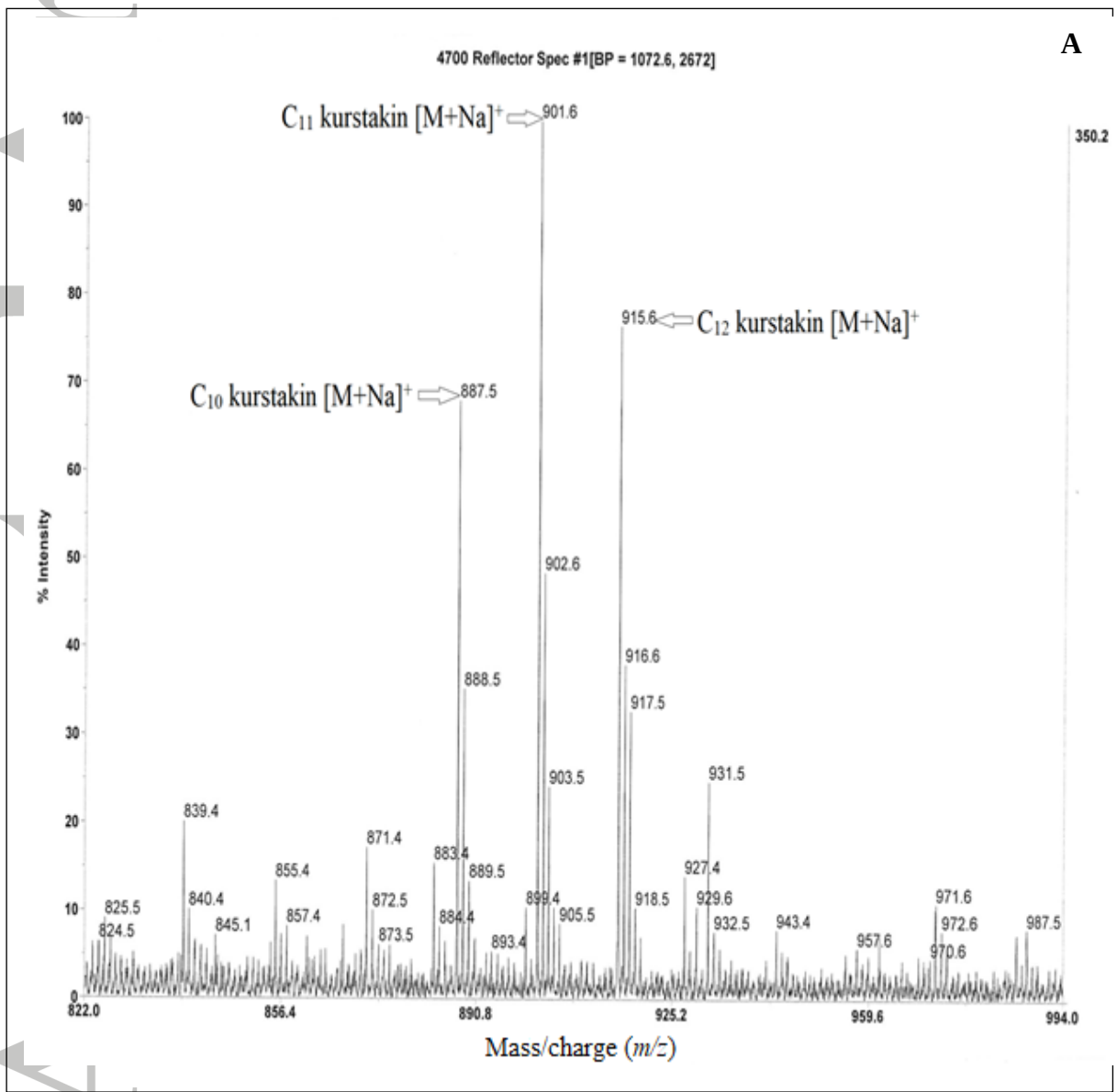
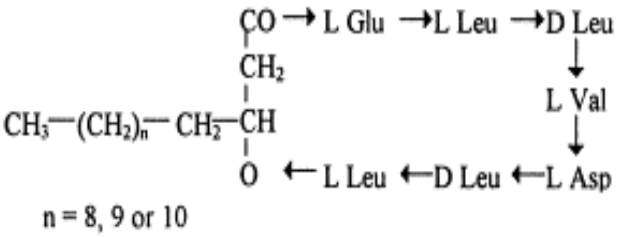


Figure. 1 Fourier transforms infrared spectrum of biosurfactants synthesized by *E. cloacae* C3 strain







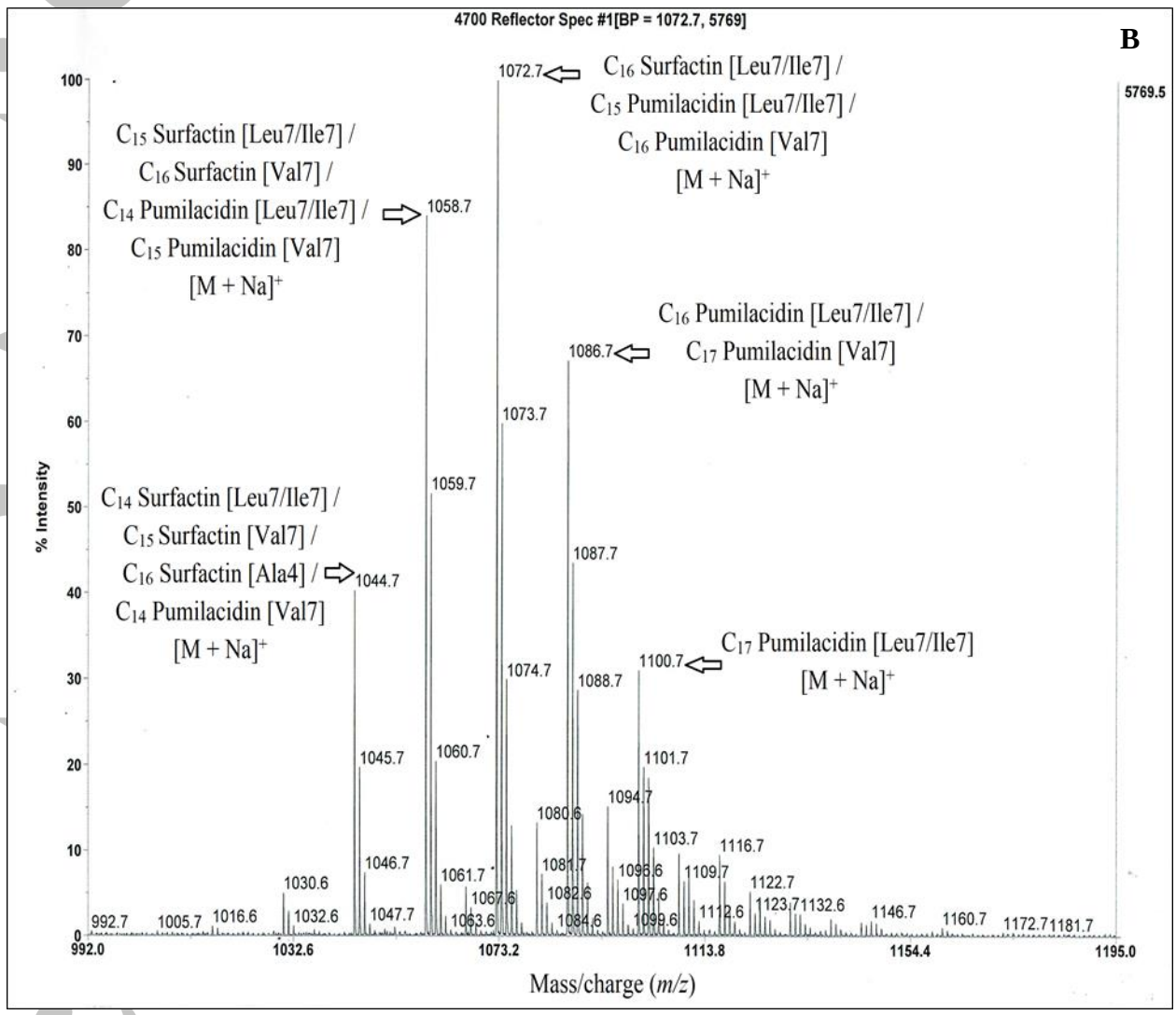


Figure. 2 Mass spectrometry (LC-MSD-TOF) analysis of lipopeptide C3 molecular signals. Spectra of lipopeptides belonging to kurstakin (A) and surfactin families (B)

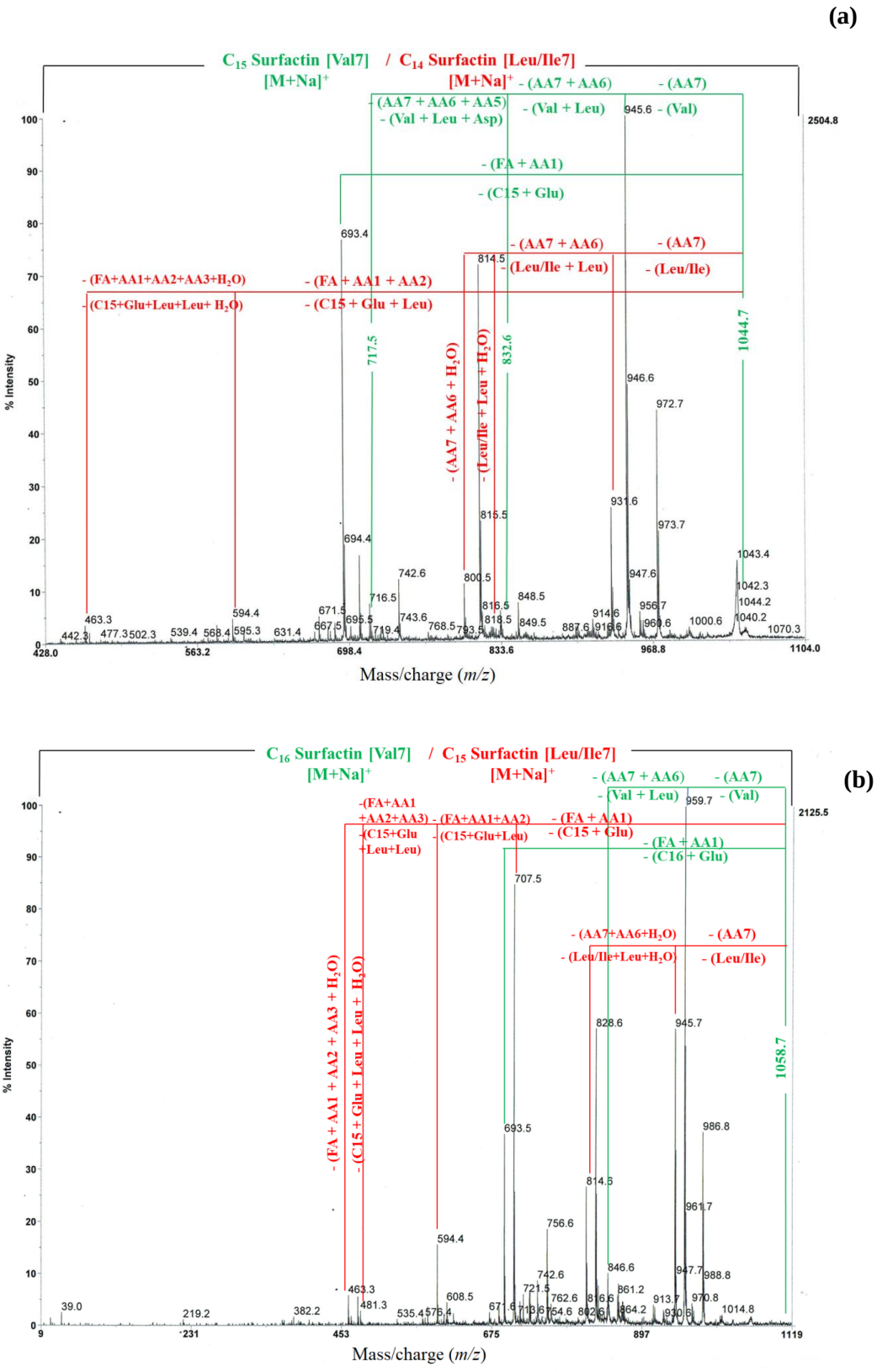


Figure. 3 Product ions spectra obtained by MALDI-TOF/TOF MS^2 of the sodiated molecules $[M+Na]^+$ of surfactin isoforms at *m/z* 1044.7 (a) and 1058.7 (b)

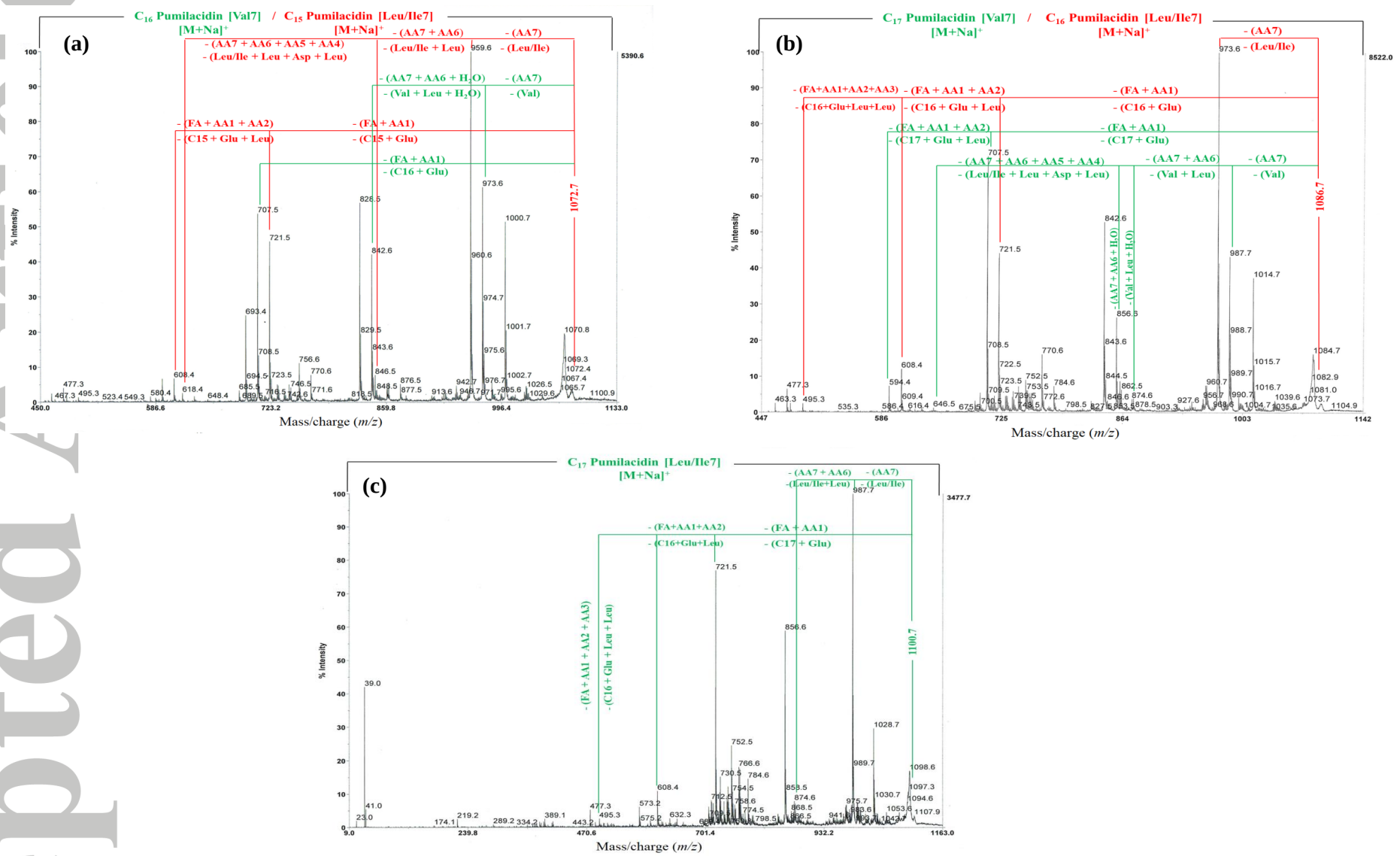
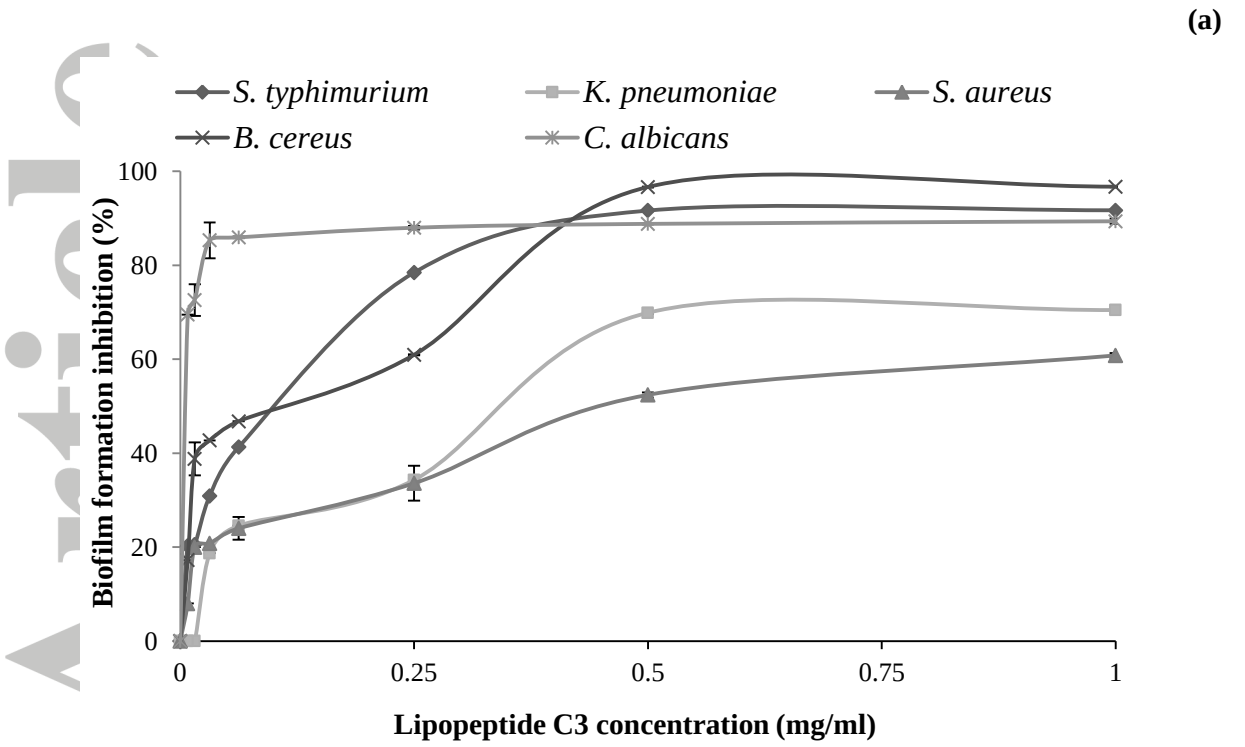


Figure. 4 Product ions spectra obtained by MALDI-TOF/TOF MS^2 of the sodiated molecules $[M+Na]^+$ of pumilacidin isoforms at *m/z* 1072.7 (a), 1086.8 (b) and 1100.7 (c)



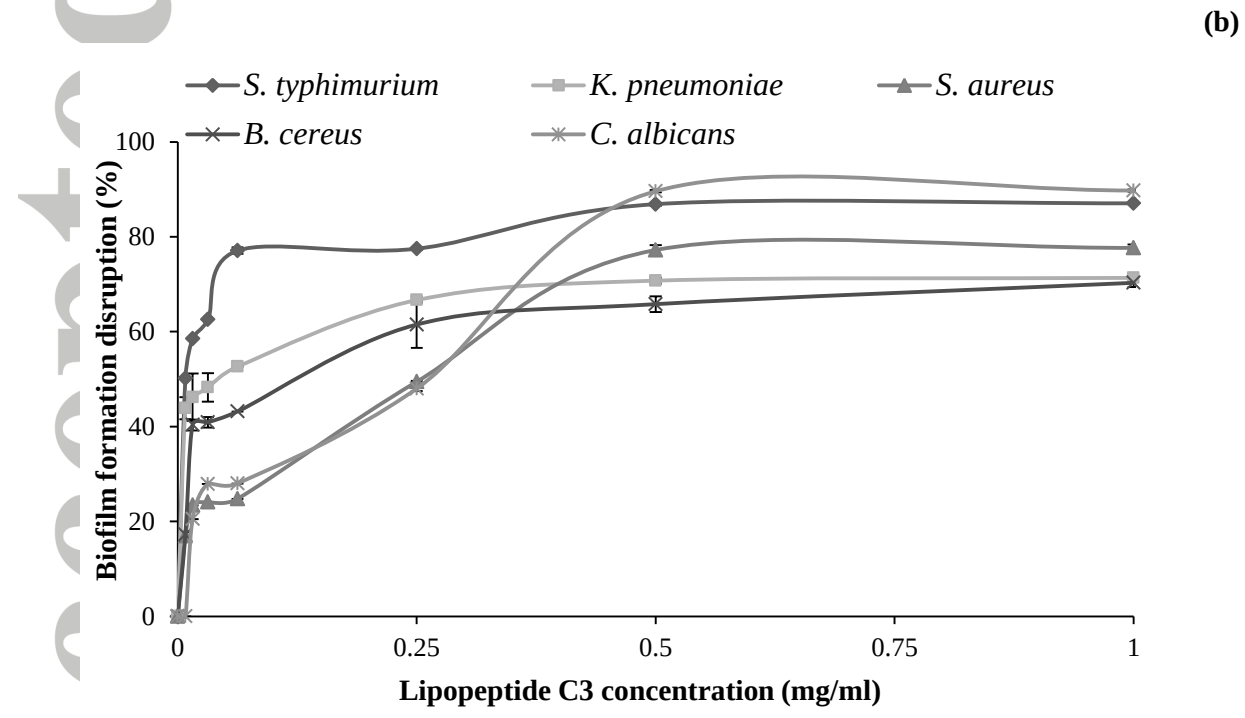


Figure. 5 Biofilm formation inhibition (a) and disruption (b) by C3 lipopeptides.