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SHORT COMMUNICATION

Purification and structural characterization of fengycin homologues produced by *Bacillus subtilis* LSFM-05 grown on raw glycerol

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Abstract Raw glycerol is a byproduct of biodiesel production that currently has low to negative value for biodiesel producers. One option for increasing the value of raw glycerol is to use it as a feedstock for microbial production. *Bacillus subtilis* LSFM 05 was used for the production of fengycin in a mineral medium containing raw glycerol as the sole carbon source. Fengycin was isolated by acid precipitation at pH 2 and purified by silica gel column chromatography and characterized using electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) with collision-induced dissociation (CID). The mass spectrum revealed the presence of the ions of *m/z* 1,435.7, 1,449.9, 1,463.8, 1,477.8, 1,491.8 and 1,505.8, which were further fragmented by ESI-MS/MS. The CID profile showed the presence of a series of ions (*m/z* 1,080 and 966) and (*m/z* 1,108 and 994) that represented the different fengycin homologues A and B, respectively. Fengycin homologues A and B are variants that differ at position 6 of the peptide moiety, having either Ala or Val residues, respectively. Mass spectrometry analyses identified four fengycin A and three fengycin B variants with fatty acid components containing 14–17 carbons. These results demonstrate that raw glycerol can be used as feedstock to produce fengycin, and additional work should focus on the optimization of process conditions to increase productivity.

Keywords *Bacillus subtilis* · Fengycin · Biosurfactant · Raw glycerol · ESI FT-ICR mass spectrometry

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Introduction

Biosurfactants are of interest in comparison to chemical surfactants due to their high level of activity, ability to be produced from renewable feedstocks, and high degree of biodegradability. However, biosurfactants have not yet been employed extensively in industry because their production is not optimized [1, 30]. Among the reasons they have not yet been commercialized extensively is the high costs of feedstocks [10, 21, 25]. Immiscible carbon sources have been traditionally used to produce biosurfactants and bioemulsifiers [3, 18, 25, 26], however, *Bacillus* strains are able to produce biosurfactants from water soluble substrates [2, 12, 22, 31]. Water-soluble substrates are preferred because single-phase fermentation is simpler than biphasic fermentation [23]. Low-cost carbon sources that have been used for biosurfactant production by microorganisms include raw glycerol [24], sludge palm oil [25], cassava wastewater [2], waste sunflower-oil [3], vegetable oil refinery waste [26], molasses and whey [16]. Raw glycerol (also known as crude glycerol) is a by-product of the biodiesel industry generated from the transesterification of vegetables oils and animal fats [11, 32], representing 10% (wt) of the biodiesel produced [9, 15]. However, raw

glycerol is currently of low value due to the presence of impurities.

According to Du et al. [11], the global biodiesel market is estimated to reach 37 billion gal by 2016 growing at an annual average of 42%. Brazil is among the largest producers and consumers of biodiesel in the world with an annual production in 2010 of 2.4 billion liters and an installed capacity in the same year of 5.8 billion liters, according to the Brazilian National Agency for Petroleum, Natural Gas and Biofuel (ANP) [5]. Any increase in biodiesel production will result in a concomitant increase in raw glycerol production and is also expected to result in decrease in the cost of raw glycerol [43]. This increase in raw glycerol production has increased interest in new and innovative uses for raw glycerol [28, 32], and is currently an important component of the future vision of biodiesel producing regions such as Brazilian.

Despite the wide range of uses for purified glycerol in food, pharmaceuticals, cosmetics, and many other products, it is frequently too costly to refine the raw glycerol to a high purity, especially for medium and small biodiesel producers [28]. If the glycerol it not purified, it typically must be disposed of in some fashion, which adds cost to biodiesel production [9, 32]. Therefore, innovative uses for raw glycerol need to be investigated for value-added applications. The use of raw glycerol as renewable substrate in industrial microbiology has been proposed as one of the solutions to this problem [32]. The ability to use raw glycerol as a substrate for the production of biosurfactants could provide a significant financial advantage against the use of pure glycerol. Morita et al. [24] reported the use of raw glycerol for production of the mannosylerythitol lipids by *Pseudozyma antarctica* JCM 10317^T and Rooney et al. [34] isolated a rhamnolipid producing microorganism from soil obtained from a biodiesel facility, on the basis of the ability to grown on glycerol as a sole carbon source. However, to the best of our knowledge, fengycin production by *Bacillus* sp. using raw glycerol as the sole carbon source has not yet been reported.

Bacillus subtilis strains produce a spectrum of lipopeptides that are powerful biosurfactants and have potent antimicrobial, antiviral and antitumor activities [36]. Three families of lipopeptides are known to be produced by *Bacillus*, all of which contain a variable cyclic amino acid portion attached to a variable β-amino or β-hydroxy fatty acid. The most studied family is the surfactins, which may be the most powerful biosurfactants known and also exhibit antiviral and antifungal activity, and contain heptapeptides attached to a β-hydroxy fatty acid with carbon number in the range of 13–15 [13, 19, 37]. The mycosubtilin, iturin and bacillomycin family, contain heptapeptides attached to β-amino fatty acids and exhibit strong antifungal activity [7, 33, 44]. Fengycins represent the third family of *Bacillus* lipopeptide surfactants and, including the related plipastatin. These surfactants are decapeptides containing ornithine attached to a β-hydroxy fatty acid, and in addition to surfactant activity also show powerful antimicrobial activity, particularly against filamentous fungi, making them of interest to agriculture industry for use in the control of plant pathogens [14, 33].

Usually, several fengycin homologues coexist in the fermentation culture of surfactant producing *Bacillus subtilis* strains [27, 35, 38]. Fengycin is a mixture of isoforms divided into two groups, A and B, based on the amino acid sequence of the peptide moiety. In fengycin A isoforms the peptide moiety consists of the sequence L-Glu, D-Orn, L-Tyr, D-Thr, L-Glu, D-Ala, L-Pro, L-Gln, D-Tyr and L-Ile, while in fengycin B isoforms Ala at position 6 is replaced by Val [4, 6, 38, 41]. In this work we demonstrated the production of fengycins by *Bacillus subtilis* LSFM 05 using raw glycerol as the sole carbon source. In addition, we purified and analyzed the structure of the fengycins produced.

Materials and methods

Microorganism and culture conditions

Bacillus subtilis LSFM-05 was used in this study and was isolated from soil with a history of contamination with hydrocarbons located at a petroleum refinery in Paulínia, São Paulo State, Brazil, and is maintained in the Culture Collection of the Systematic and Physiology Microbial Laboratory (FEA-UNICAMP). The growth and production medium used was a mineral salt medium: 3.0 g l^{-1} of $NaNO_3$; 1.0 g l^{-1} of KH_2PO_4; 0.1 g l^{-1} of NaCl; 0.5 g l^{-1} of $MgSO_4.7H_2O$ and 1 ml of vitamins stock solution (2.0 mg l^{-1} folic acid, 10 mg l^{-1} pyridoxine, 5.0 mg l^{-1} riboflavin, 5.0 mg l^{-1} thiamine, 5.0 mg l^{-1} nicotinic acid, 5.0 mg l^{-1} pantothenic acid, 0.1 mg l^{-1} cyanocobalamin, 5.0 mg l^{-1} ρ-aminobenzoic acid, 5.0 mg l^{-1} thioctic acid and 2.0 mg l^{-1} biotin) with initial pH 6.8–7.2. Fermentation was carried out in 15-l bench-top fermentor (Bioflo 3,000 New Brunswick Scientific) containing 10 l of basal salts medium plus 5% (v/v) raw glycerol as the sole carbon source. The glycerol sample was donated by the biodiesel producer Granol (Anápolis, Goiás, Brazil) and stored at 4°C.

The fermentation was carried out at 32°C with stirring at 250 rpm and aeration of 0.5 vvm for 72 h, without the addition of chemical antifoam. The pH was not controlled during the fermentation. Fengycin production was carried out in three independent replicates. Samples were collected at 12-h intervals for analysis of cell density and glycerol concentration. The cells were removed by centrifugation at

18,000 × *g* for 10 min at 4°C and the glycerol concentration determined by HPLC using a Shimadzu Chromatograph model CR-21 equipped with a Supercogel C-610H column conditioned at 75°C and an isocratic mobile phase (0.1% H_2SO_4) [20]. Microbial growth was determined by measuring the cell dry weight. At the indicated times, 10-ml samples were withdrawn and centrifuged at 18, 000 × *g* for 10 min at 4°C, washing twice with saline solution (0.85%, w/v). The biomass obtained was re-suspended in distilled water, dried overnight at 100°C, and then weighed.

Isolation of the fengycin homologues

The raw biosurfactant extract was recovered from the foam overflow formed during fermentation. Foam overflow was collected in a vessel connected to the air-exhaust line. The foam was centrifuged at 18,000 × *g* for 15 min at 4°C to remove cells and the supernatant was acidified with concentrated HCl to pH 2.0 and incubated for 24 h at 4°C to precipitate the surfactants [8]. The raw biosurfactant was separated from the acidified supernatant by centrifugation at 18,000 × *g* for 15 min at 4°C, washed twice with acidified water, freeze-dried using a Dura Dry-FTS-System, and weighed on an analytical balance.

Purification of the fengycin homologues on an analytical scale

Fengycin was purified by column chromatography, using open columns (40 × 5 cm), filled with Acros Organics silica gel (0.03–0.07 mm, 60 Å) as the stationary phase. One gram of the raw extract was dissolved in chloroform/methanol (2:1) and added to the column. The samples were eluted using a mixture composed of chloroform, methanol, and an aqueous solution of 28% (v/v) ammonium hydroxide with increasing polarities as follows: $CHCl_3$/CH_3OH/NH_4OH (80:20:4) (v/v/v), $CHCl_3$/CH_3OH/NH_4OH (75:25:4) (v/v/v) and $CHCl_3$/CH_3OH/NH_4OH (65:35:5) (v/v/v). Ten-ml fractions were collected, and the presence of biosurfactant monitored by TLC using silica gel 60 plates (Macherey–Nagel-Alugram-SilG/UV254) as the stationary phase and $CHCl_3$/CH_3OH/NH_4OH (75:25:4) (v/v/v) as the mobile phase. The plates were developed by spraying with distilled water and incubating at 100°C, which produced white spots where surfactant was present. Fengycin was distinguished from surfactin as a surfactant spot with a migration R*f* different from that of a surfactin standard (Sigma-Aldrich). The organic fractions containing fengycin were pooled together in a round volumetric flask and the organic solvent evaporated off under reduced pressure in a rotary evaporator (Eyla-A-3S) followed by re-suspension in deionized water, freezing, and freeze-drying of the purified biosurfactant. This fengycin-containing fraction was used for IR spectroscopy and ESI mass spectrometry analysis.

Infrared analysis

Samples for infrared analysis were prepared by mixing approximately 1.0 mg of purified biosurfactant with 100 mg of KBr and pressing the mixture into a pellet for

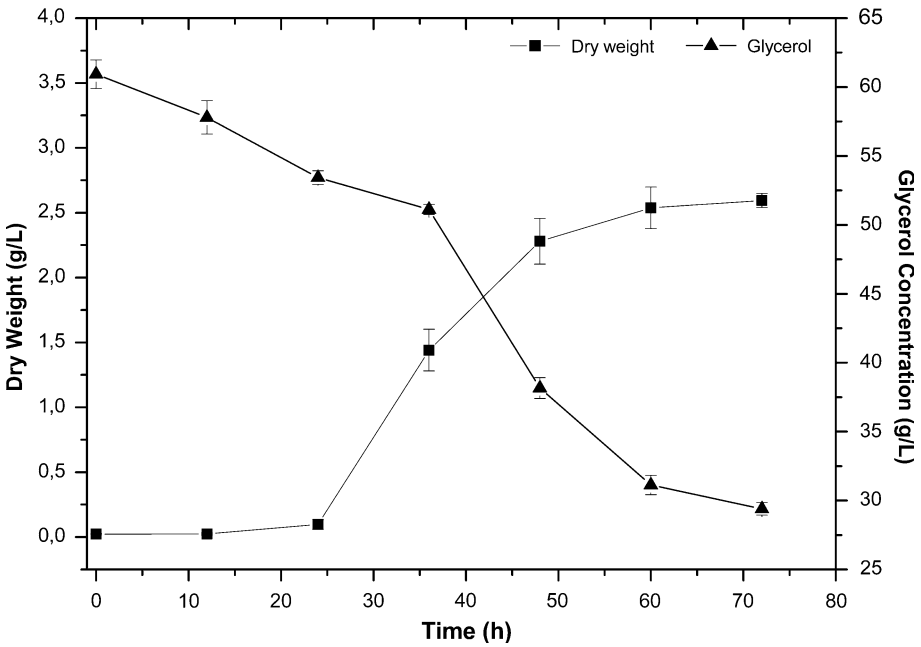

Fig. 1 Growth and glycerol consumption profile of *B. subtilis* LSFM-05 grown in mineral medium supplemented with 5% (v/v) of raw glycerol as substrate at 32°C for 72 h

2–3 min at 20,000 psi. The infrared spectra of the pellets were obtained using a FT-IR (FTLA2000) spectrometer from 400 to 4,000 wavenumbers (cm^{-1}).

Mass spectrometry analysis ESI-FT-ICR/MS

A sample of a purified biosurfactant (1 mg) was dissolved in 1 ml of methanol. For analysis in the positive-ion mode, a total of 1 µl of a 0.1% (v/v) aqueous solution of formic acid (27 µM final concentration) was added to facilitate protonation of the basic nitrogen compounds yielding $[M + H]^+$ ions. A direct infusion automated chip-based nano-ESI Triversa NanoMate 100 system (Advion BioSciences, Ithaca, NY, USA) was used in both the positive and negative ion modes. Samples were loaded into 96-well plates (total volume of 100 µl in each well) and analyzed by a 7.2T LTQ FT Ultra mass spectrometer (Thermo Scientific, Bremen, Germany). ESI conditions were: gas pressure of 0.3 psi and capillary voltage of 1.55 kV. Mass spectra are the result of over 100 microscans processed via the Xcalibur 2.0 software (ThermoScientific, Bremen, Germany).

Results and discussion

Biosurfactant production

Bacillus subtilis LSFM-05 was grown on 5% (v/v) raw glycerol as the sole carbon source for 72 h. Figure 1 shows the average values of cell growth and glycerol consumption by *Bacillus subtilis* LSFM-05 over the course of the fermentation. The growth of *B. subtilis* LSFM-05 on raw glycerol was characterized by a lag phase for 24 h followed by an exponential growth phase, which was complete after 48 h of incubation. The final biomass achieved was 2.59 ± 0.05 g/l dry cell weight after 72 h of fermentation. Maximum glycerol consumption occurred in the interval from 36 to 48 h and at the end of the fermentation 52% of the raw glycerol had been consumed. The initial pH of the culture broth was 6.9 ± 0.1 and the final pH was 8 ± 0.18. An increase in pH values from to 6.8 to 7.7 was also observed by Reis et al. [31] for *B. subtilis* cultivated in mineral medium containing purified glycerol as the carbon source. Fox and Bala [12] also reported an increase in pH values from 6.2 to 8.5 in different un-buffered potato media.

A total volume of 3.91 ± 0.36 liters of foam overflow was collected during the fermentative process and the overall biosurfactant yield in the acid precipitate of the colleted foam was 0.93 ± 0.4 g/l. The surface tension of the culture broth at the beginning of the fermentation was 47.5 ± 1.2 mN/m. In contrast, the surface tension of the cell free foam was 31.0 ± 1.1 mN/m, demonstrating the presence of surfactants. The surface tension of ten and 100 times-diluted cell-free foam supernatant was 32.7 ± 0.9 and 41.6 ± 1.4 mN/m, respectively, indicating that the biosurfactant concentration in the foam was at least ten times its CMC. There was no decrease in surface activity in the medium that remained in the vessel at the end of fermentation indicating that virtually all of the surfactant partitioned into the foam. Surfactant was not isolated from the culture medium remaining in the fermentor.

The yield of biosurfactant in the foam reached a maximum value of 1.37 ± 0.13 g/l for the 12-h period ending 60 h after incubation, whereas the overall foam production peaked at 2.4 ± 0.17 l for the 12-h period ending 48 h after inoculation. Maximum surfactant and foam yield therefore correspond to the maximum growth phase of the culture, demonstrating a growth related production of surfactant. The productivity of surfactant corresponding to the maximum surfactant yield was 11.42 ± 1.1 mg/l/h.

A similar foam fractionation approach was used by Cooper et al. [8] to purify biosurfactants produced by *B. subtilis*. Their study showed a biosurfactant yield from the foam of 0.8 g/l when the *B. subtilis* was cultivated in medium containing glucose as the carbon source. Makkar and Cameotra [22] reported the production yield about 4.5 g/l biosurfactant in acid precipitates of collected foam produced by *B. subtilis* MTCC 2423. The yield showed in this report is still low compared with the values presented by Makkar and Cameotra [22], however, it should be able to be improved using a continuous or feed-batch

Fig. 2 Photographic negative of the purified surface active components separated on silica gel TLC plates: SD- surfactin standard and F1.1 and F1.2 eluates from the adsorption chromatography column containing fengycin produced by *Bacillus subtilis* LSFM-05

fermentation process and other modification that can be identified in optimization studies.

Following the initial isolation by acid precipitation, the surfactant was purified by column chromatography as described in the Materials and methods section. The productivity of the purified fengycin homologues was 30 mg per liter of collected foam.

Thin-layer chromatographic and Fourier transformed-infrared (FT-IR) spectrum of the purified fengycin

The acid treatment of the foam yielded a brown-colored precipitate containing the biosurfactant. Surfactants present in the precipitate were visualized by spraying the developed TLC plates with distilled water, which produced white spots in the presence of biosurfactant. Fengycin was distinguished from surfactin based on the difference in it's R*f* (retention factor) in comparison to commercially available surfactin (Sigma-Aldrich) (Fig. 2). The FT-IR spectrum of fengycin produced by *Bacillus subtilis* LSFM-05 showed a strong absorption band at 3,300 cm^{-1} resulting from N–H stretching. The band at 1,650 cm^{-1} is attributable to the stretching of a N-CO group (amide bond) and the band at 1,535 cm^{-1} is consistent with the deformation mode of a N–H bond combined with a C–N stretching band, all of which indicate the presence of a peptide component. The presence of an aliphatic chain is indicated by the typical C–H stretching modes at 2,970–2,850 cm^{-1} and 1,450–1,380 cm^{-1}. Similar stretching modes were found in previous studies of lipopeptide surfactants [16, 29, 42]. These results indicate that the purified biosurfactant isolated by column chromatography contained aliphatic and peptide moieties. In addition, the adsorption band at 1,730 cm^{-1} is consistent with a lactone carbonyl, which is common to lipopeptide biosurfactants [39, 42]. The band at 1,730 cm^{-1} was also observed in a fengycin analyzed by Pueyo et al. [29].

Mass spectrometry

The mass spectrum of the purified surfactant shows a set of ions $[M + H]^+$ ions of *m/z* 1,435.7, 1,449.9, 1,463.8, 1,477.8, 1,491.84, and 1,505.8 consistent with the mass data obtained from different fengycin homologues in previous studies (Fig. 3) [4, 17, 38, 42]. The ions of greatest abundances in the sample were those of *m/z* 1,463.8, 1,477.8, and 1,491.8, whereas the ion of *m/z* 1,463.8 was the most predominant (Fig. 3). As shown by the CID profile of the ion of *m/z* 1,463.8 (Fig. 4), the amino acid moiety contained Ala, Thr, Glu, Pro, Ile, Tyr, Orn, Gln in molar ratios (1:1:2:1:1:2:1:1) and ring opening during fragmentation was preferentially between alanine and proline as shown by the fragments of *m/z* 226.1, 389.1,

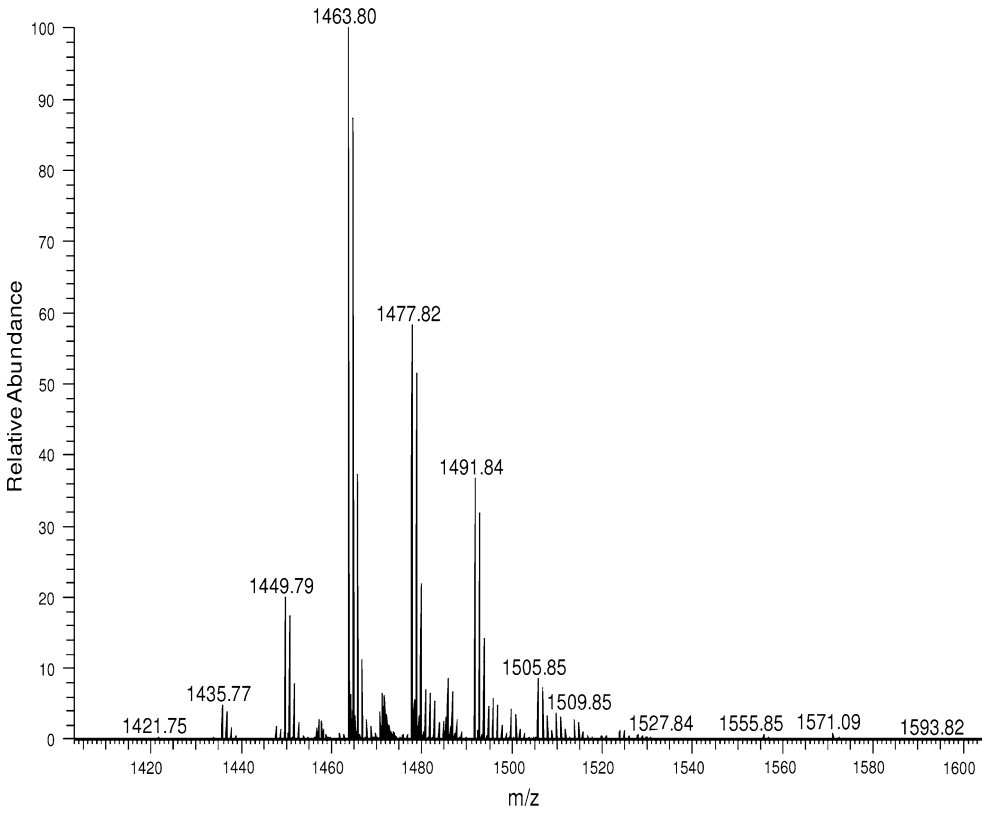

Fig. 3 Mass spectrometry (ESI-MS) of the biosurfactant purified by column chromatography

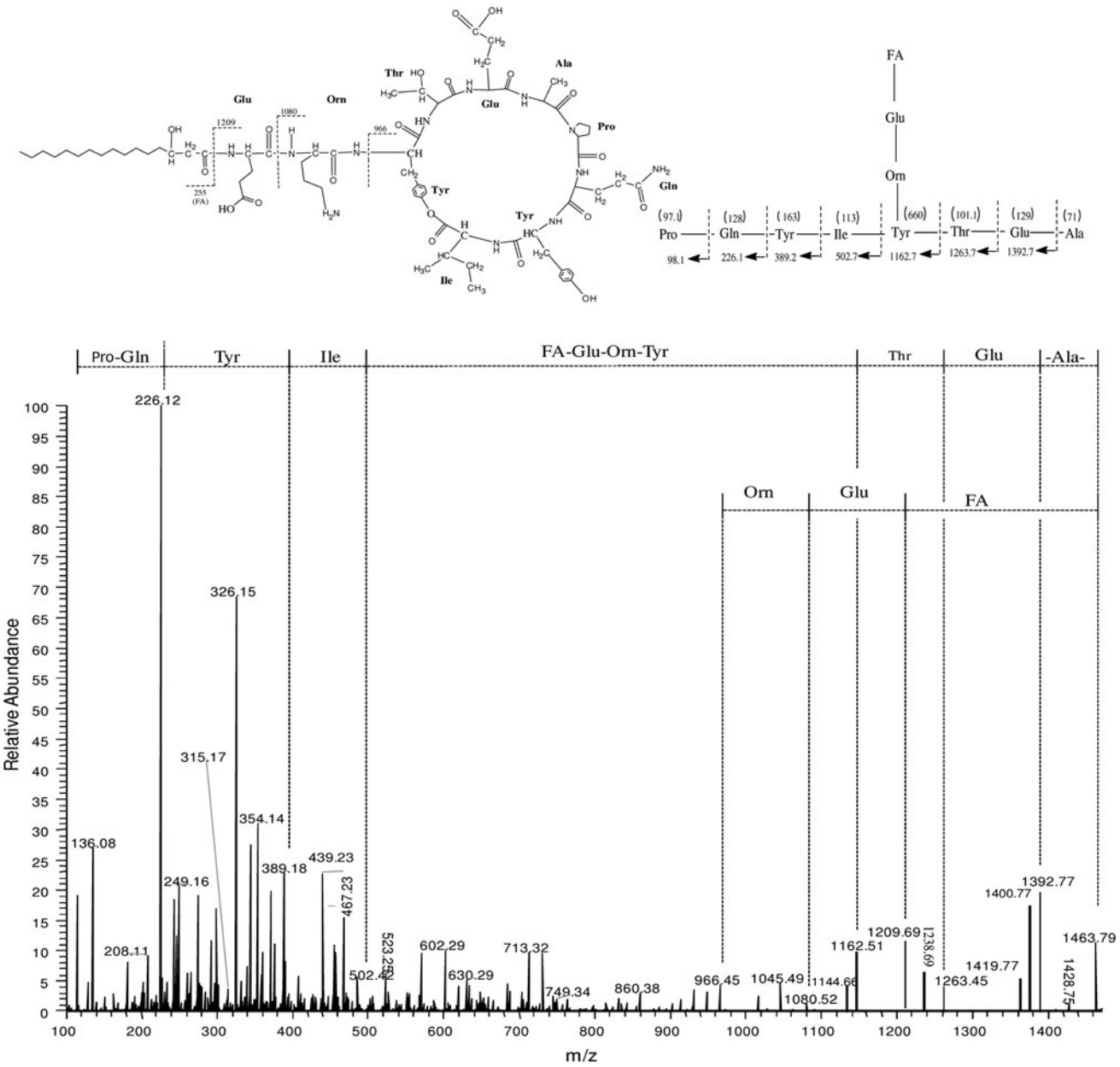

Fig. 4 ESI-MS-MS for CID of the protonated molecule of *m/z* 1,463.8 showing the peptide and fatty acid composition of this fengycin homologue

502.1, 1,162.7, 1,263.7, and 1,392.7. Tsuge et al. [40] characterized a plipastatin lipopeptide produced by *Bacillus subtilis* that showed the same amino acid composition as fengycin except for the replacement of the glutamic acid in fengycin with a glutamine in plipastatin. The fragmentation pattern of the ion of *m/z* 1,463.8 is consistent with the fatty acid portion containing 16 carbon atoms and the fragmentation product of *m/z* 1,209 corresponds to the neutral loss of the fatty acid chain (Fig 4).

The fragmentation profile of the ions of *m/z* 1,463.8, 1,435.7, and 1,449.9 contained product ions of *m/z* 966 and 1,080. This pair of ions was described previously by Bie et al. [4] and Hu et al. [14] as those originating from fengycin A and can be explained by neutral losses of (fatty acid-Glu) and (fatty acid-Glu-Orn) from the N-terminal segment, respectively. Figure 4 shows the pair of ions of *m/z* 966 and 1,080 from the fragmentation of the ion of *m/z* 1,463.8.

The fragmentation product ions of *m/z* 994 and 1,108 were found in the CID spectra of the precursor ions of *m/z* 1,505.85 and 1,491.84 (Fig. 5a). The pair of ions of *m/z* 994 and 1,108 can be attributed to neutral losses of fatty acid-Glu and fatty acid-Glu-Orn, respectively, from the N-terminal segment of fengycin B [4, 14, 38]. The ions are 28 Da heavier than the corresponding ions from fengycin

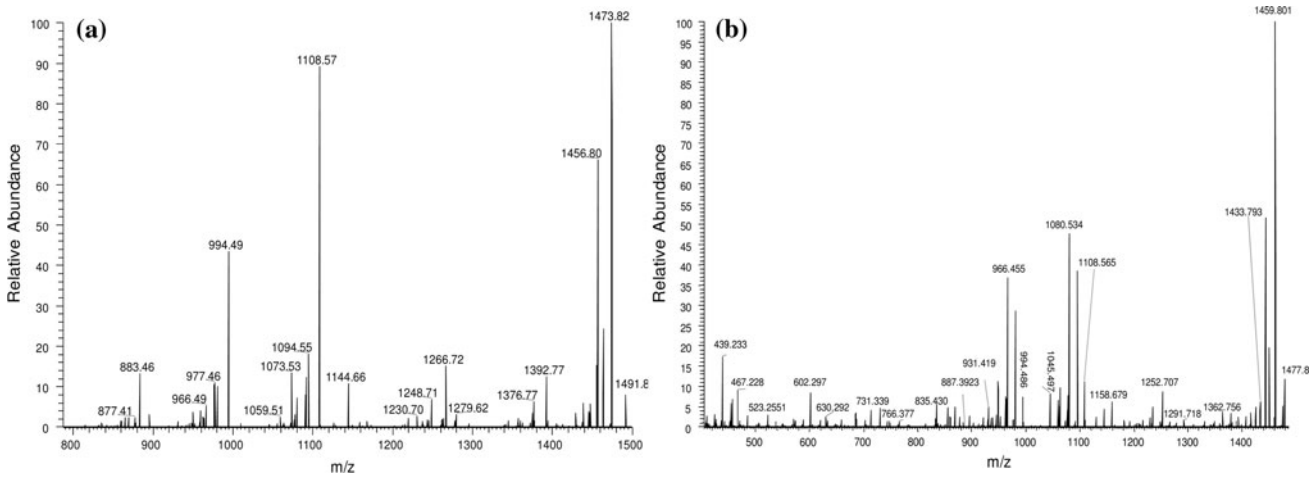

Fig. 5 **a** ESI-MS-MS for CID of the protonated molecule of *m/z* 1,491.8 showing the fragments of *m/z* 1,108 and 994 and **b** of *m/z* 1,477.8 showing the fragments of *m/z* 1,080, 1,108, 966 and 994

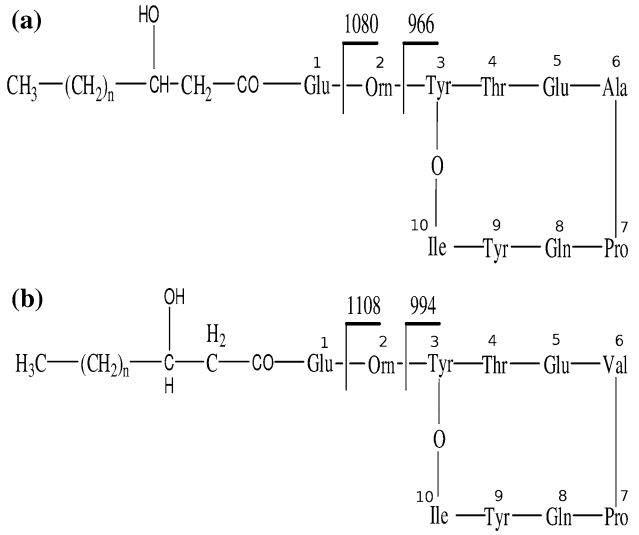

Fig. 6 Primary structure of **a** fengycin homologue A and **b** fengycin homologue B

A, reflecting the presence of Ala in the lactone ring of fengycin A and Val in the lactone ring of fengycin B [4, 41] as illustrated in Fig. 6a and b, respectively. Thus, ions of *m/z* 1,435.7, 1,449.9, and 1,463.8 were assigned to fengycin A and the ions of *m/z* 1,491.8 and 1,505.8 to fengycin B (Table 1). Interestingly, the pairs of fragment ions of *m/z* 966 and 1,080, and *m/z* 994 and 1,108, were both observed for the precursor ion of *m/z* 1,477.9 (Fig. 5b), indicating that both fengycins A and B contributed to the production of the ion of *m/z* 1,477.9 [41]. This association was also found by Wang et al. [41] and is attributed to the difference in the length of the fatty acid component of fengycin isoforms within each homologue group (A and B), with isoforms differing by multiples of 14 Da corresponding to one CH_2 group. Table 1 shows that

Table 1 Assignment of fengycin homologues characterized by the CID profiles of their protonated molecules

Ion (*m/z*)	Productions	Assignment	Amino acid at position 6
1,435.7	966	C-14 fengycin A	Ala
1,449.7	966	C-15 fengycin A	Ala
1,463.8	966	C-16 fengycin A	Ala
1,477.8	966	C-17 fengycin A	Ala
1,477.8	994	C-15 fengycin B	Val
1,491.8	994	C-16 fengycin B	Val
1,505.8	994	C-17 fengycin B	Val

the length of the fatty acid component can vary from C-14 to C-17 in both fengycins A and B. As a result the molecular weight of isoforms of fengycin A can have the same molecular weight as that of an isoform of fengycin B with a fatty acid tail of two CH_2 groups shorter, equivalent to the molecular weight difference between Val and Ala (28 Da) [41] highlighting the need for mass spectrometry to distinguish between fengycin A and B homologues.

Conclusions

Our results demonstrate that raw glycerol can be used as an renewable carbon source for the production of fengycin by *Bacillus* and may provide a low cost feedstock for the large-scale production of this surfactant, especially in countries such as Brazil that produce large amounts of biodiesel from vegetable oils and waste fats oils from cooking and meat production. To our knowledge, this is the first report on the production of fengycin by a *Bacillus subtilis* strain using raw glycerol obtained from a biodiesel factory as feedstock.

ESI FT-ICR mass spectrometry proved to be a rapid method to identify and distinguish between fengycin homologue groups A and B. Using this technique, fengycin homologues A and B can be quickly identified based on the presence of the pair of ions of *m/z* 1,080 and 966, and *m/z* 1,108 and 994, respectively.

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