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# Production and characterization of microbial biosurfactants for potential use in oil-spill remediation

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ABSTRACT

Two biosurfactants, surfactin and fatty acyl-glutamate, were produced from genetically-modified strains of *Bacillus subtilis* on 2% glucose and mineral salts media in shake-flasks and bioreactors. Biosurfactant synthesis ceased when the main carbohydrate source was completely depleted. Surfactin titers were ~30-fold higher than fatty acyl-glutamate in the same medium. When bacteria were grown in large aerated bioreactors, biosurfactants mostly partitioned to the foam fraction, which was recovered. Dispersion effectiveness of surfactin and fatty acyl-glutamate was evaluated by measuring the critical micelle concentration (CMC) and dispersant-to-oil ratio (DOR). The CMC values for surfactin and fatty acyl-glutamate in double deionized distilled water were 0.015 and 0.10 g/L, respectively. However, CMC values were higher, 0.02 and 0.4 g/L for surfactin and fatty acyl-glutamate, respectively, in 12 parts per trillion (ppt) Instant Ocean® sea salt, which has been partly attributed to saline-induced conformational changes in the solvated ionic species of the biosurfactants. The DORs for surfactin and fatty acyl-glutamate were 1:96 and 1:12, respectively, in water. In Instant Ocean® solutions containing 12 ppt sea salt, these decreased to 1:30 and 1:4, respectively, suggesting reduction in oil dispersing efficiency of both surfactants in saline. Surfactant toxicities were assessed using the Gulf killifish, *Fundulus grandis*, which is common in estuarine habitats of the Gulf of Mexico. Surfactin was 10-fold more toxic than fatty acyl-glutamate. A commercial surfactant, sodium laurel sulfate, had intermediate toxicity. Raising the salinity from 5 to 25 ppt increased the toxicity of all three surfactants; however, the increase was the lowest for fatty acyl-glutamate.


## 1. Introduction

There is a growing demand for "green" and non-toxic dispersants for oil spill remediation. Due to the environmental and health risks from synthetic chemicals, surfactants from biological sources are favored for their biodegradability and potentially lower toxicity. Currently, surfactants are synthesized from petroleum-based hydrocarbons and are used in foods, medicines, industrial applications [1,2], and waste remediation [3,4]. It is estimated that current global surfactant production consumes about 7.4 billion kg of petrochemical intermediates and emits about 31.6 billion kg of $CO_2$ [5,6].

Biosurfactants include surface-active chemicals synthesized by a wide variety of microorganisms [7–9]. Examples include iturins, esperine, mycosubtilin and surfactin. These are cyclic lipopeptides produced by *Bacillus* sp., which have interesting physiological, biocidal, physicochemical, and surface-active properties. Identification and characterization of effective, environmentally friendly, and low-cost biodispersants is of critical importance. The selection of a dispersant for oil spills is influenced by its efficacy and environmental impact. Economic production of biodispersants from low-cost biological feedstocks with efficient green recovery methods will improve their acceptability, augment their use, and reduce or eliminate the need for synthetic dispersants.

Surfactin is a well-known lipopeptide with surface-active properties and was first co-produced with iturinic lipopeptides using

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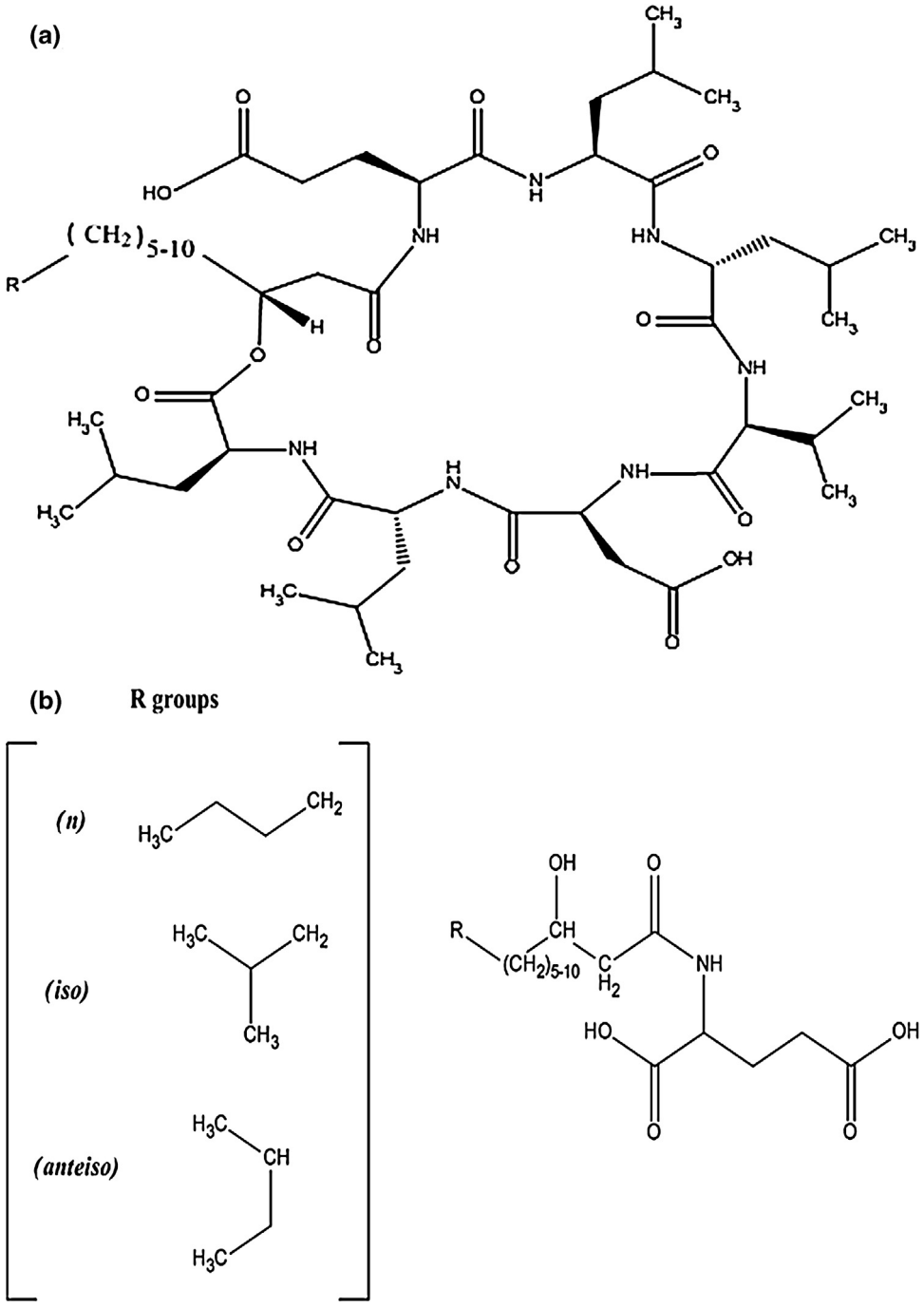


**Fig. 1.** Structures of lipopeptide biosurfactants: (a) surfactin and (b) FA-Glu. The variability in the fatty acid chain length of both surfactants is responsible for the presence of several isoforms of different molecular weight. The molecular weights of surfactin's isoforms are 978.59, 992.59, 1006.62, 1020.64 and 1034.64 and 1048.69; those of FA-Glu are 344.07, 358.07, 372.08, 386.08, 400.09 and 414.09.

*Bacillus subtilis* [10,11]. It consists of a long-chain β-hydroxy fatty acid joined by an amide bond to the glutamic acid residue of a heptapeptide having the chiral sequence: L-Glu/L-Leu/D-Leu/L-Val/L-Asp/D-Leu/L-Leu (Fig. 1a). As its fatty acid chain has from 12 to 17 carbons, surfactin exists as a population of surfactant isoforms that range in molecular weight from 978.59 to 1048.69 and differ by 14 amu, the molecular weight of a methylene (—$CH_2$—) residue. The production of surfactin and similar lipopeptides from *Bacillus* species is reported in the literature. Liu et al. showed with *B. subtilis TD7* that the type of amino acid present in the growth medium resulted in surfactants with certain characteristics: for example, inclusion of Arg, Gln or Val in medium resulted in predominance of surfactin molecules with even-numbered carbon chains, whereas when Cys, His, Ile, Leu, Met, Ser or Thr were included, surfactin molecules with odd-numbered carbon chains predominated [12]. de Faria and co-workers (2011) used *B. subtilis LSFM-05* to produce a surfactin variant with an amino acid sequence of GluOMe-Leu-Leu-Asp-Val-Leu-Leu and a C14 fatty acid moiety from glycerin derived from biodiesel production.

The CMC and emulsification index ($E_{24}$) of the surfactant are also reported and compared with those of sodium dodecyl sulfate (SDS) and Triton X [13]. Liu and co-workers (2010) determined CMCs and surface tensions in phosphate buffered saline (PBS) of nC14- and anteiso C15-surfactins produced by *Bacillus velezensis*

strain H3 isolated from marine sediment [14]. Rangarajan et al. demonstrated the importance of trace metals in microbial production of lipopeptides by showing that addition of $Fe^{2+}$ to the growth medium increased surfactin titers from *Bacillus megaterium* [15].

An important concern for biosurfactants intended for use in oil spill remediation is toxicity to aquatic organisms. This is a concern because surfactants reach the environment in the form of household products, industrial cleaners, and oil spill dispersants. The toxicity in coastal environments and the effects of salinity on surfactant toxicity are important issues as use of surfactants for oil dispersion in deep water can introduce man-made chemicals to coastal regions and rivers, which experience dynamic salinity and generally are extremely productive [16].

Surfactin is a very surface-active molecule: it reduced the surface tension of water from 72- to 27-mN/m at 20 μM [10]. However, its low solubility in aqueous systems could limit its efficacy for dispersing oil spills at sea. Modular Genetics, Inc. (Woburn, MA) has genetically engineered strains of *B. subtilis* to produce surfactin derivatives with advanced properties [6] and more favorable solubility characteristics. One such variant is fatty acyl-glutamate (FA-Glu) with β-hydroxy fatty acid chains of 12–17 carbons linked to a single glutamic acid residue by a peptide bond (Fig. 1b). Like surfactin, FA-Glu consists of several isoforms that differ by 14 amu and range in molecular weight from 344.07 to 414.09. The overall goal of the study is to develop and compare biosurfactants from various methods for their suitability as dispersants for crude oil breakup and also for food applications. This study, however, reports the synthesis of biosurfactant (surfactin and FA-Glu) by two *B.subtilis* variants from the same parent *Bacillus* strain and comparison of their surface-activity and toxicities in water and saline.

## 2. Materials and methods

### 2.1. Microorganisms and culture conditions

*B. subtilis* strains 41651 A1 and 40688 E4 were used to produce surfactin and FA-Glu, respectively, and were provided by Modular Genetics, Inc. (Woburn, MA). Both strains were modified from *B. subtilis* OKB105 by incorporating phenylalanine auxotrophy and eliminating spore forming capability. Specifics on strain properties and genetic modifications are reported elsewhere [6]. All strains were stored in 20% glycerol at −80 °C until needed in a SO-LOW Ultra Low freezer (Environmental Equipment, Cincinnati, OH). The seed medium used to maintain and propagate the strains prior to inoculation contained 6 g $Na_2HPO_4$, 3 g $KH_2PO_4$, 0.5 g NaCl, 1 g $NH_4Cl$, 3 g yeast extract, 5 g glucose and 10 g casamino acids per liter. The medium, except glucose and casamino acids, was sterilized by autoclaving at 121 °C, and then supplemented with filter-sterilized glucose and casamino acid solutions. The growth medium (termed "S-7") used for surfactant cultivation contained 100 mM potassium phosphate buffer, pH 7.5, and 100 mM $(NH_4)_2SO_4$. The medium was supplemented with glutamic acid (pH 7.0) and glucose to final concentrations of 20 mM and 111 mM (20 g/L), respectively. Per liter of S-7 medium, 10 mL of a sterile trace metal solution containing 24 mM HCl, 200 mM $MgCl_2$–$6H_2O$, 10 mM $CaCl_2$–$2H_2O$, 5 mM $MnCl_2$–$4H_2O$, 0.1 mM $ZnCl_2$ and 0.5 mM $FeCl_3$–$6H_2O$ were added, followed by 10 mL of a sterile solution containing 0.68 mg of thiamine–HCl. Glucose, glutamic acid, thiamine–HCl and trace metal solutions were separately prepared and filter-sterilized prior to use.

### 2.2. Cultivation conditions in shake-flasks and bioreactors

Inocula for shake flasks and bioreactors were prepared by preculturing the organisms overnight in seed medium at 30 °C with shaking at 160 rpm on a Model G76 Gyrotory Water Bath Shaker (New Brunswick Scientific, NJ). Growth was monitored spectrophotometrically at 650 nm. Pre-culture inocula were added to S-7 medium to produce an initial $A_{650}$ of ~0.03–0.05. Initial growth rate during log-phase was calculated using Eq. (1) and absorbance values obtained during the first 10 h. The logarithm of absorbance was plotted against time to produce a straight line as defined by Eq. (1). The slope of the line was the initial growth rate ( ).

$$\ln X = \mu t + \ln X_0 \quad (1)$$

In several of the cultivations in bioreactor, foam was produced at about 10 h. The foam subsequently filled the bioreactor headspace and exited through a sterile connecting tube to a sterile carboy. The exiting foam also removed some cells from the bioreactor; the initial microbial growth rates thus calculated are estimates with an accuracy of ±5%.

#### 2.2.1. Shake flask fermentations

FA-Glu and surfactin were produced in shake flasks. After inoculation of the S-7 fermentation broth, the flasks were incubated at 37 °C with agitation at 160 rpm. Periodically, samples were aseptically withdrawn to monitor cell growth ($A_{650}$), clarified by centrifugation and/or syringe filtration (0.22 μm), and stored at −80 °C for later analyses for soluble carbohydrate (CHO), surfactant concentration and pH change. Fermentations were carried out for 48–72 h after which the fermentation broth was clarified by centrifugation (10,000 × *g*, 15 min, 4 °C). Supernatants were stored at −20 °C until surfactant purification and characterization tests could be performed.

#### 2.2.2. Bioreactor fermentations

Growth of *B. subtilis* and surfactant production were compared under small and large scale conditions. For this reason, FA-Glu and surfactin were also produced in 5-L bioreactors programmed with a Celligen 310 Bioreactor Control System (New Brunswick Scientific, Edison, NJ) to scale up the production. For larger-scale production, a 15-L bioreactor was used (Model D525, Bioengineering AG, Wald, Switzerland. The bioreactors were charged with solutions containing potassium phosphate buffer, pH 7.5 and $(NH_4)_2SO_4$, then sterilized by autoclaving (5-L bioreactor) or sterilization-in-place (15-L bioreactor). When these had cooled, the remaining sterile S-7 medium ingredients plus inoculum were pumped to the bioreactor from an l-L carboy using a peristaltic pump. Microbial cultivations were conducted at 37 °C, 250 rpm agitation, and 2.5- and 5.0-L/min aeration for both surfactin and FA-Glu, respectively. The amount of foam produced and transferred to the carboy depended on aeration rate. For the surfactin-producing variant, the aeration rate was decreased to 2.5 L/min since at 5 L/min nearly half of the broth ended up in carboy resulting in the cell loss and reduced surfactin production during preliminary runs. Periodic samples were aseptically withdrawn to monitor growth ($A_{650}$), pH and carbohydrate concentrations. Foam resulting from biosurfactant production, agitation and aeration filled the bioreactor headspace after 6–8 h. Continued aeration carried the surfactant-enriched foam out of the bioreactor through a sterile connecting tube to a sterile 10-L carboy. After 32–48 h, the collapsed foam was recovered as liquid and clarified by centrifugation as described above, and the supernatant stored at −20 °C until surfactant purification and characterization tests could be performed.

#### 2.2.3. Purification of surfactants

Surfactin and FA-Glu were purified by proprietary techniques that used only water as solvent. Purities (>95%) were confirmed by comparing quantitative LC/MS measurements with dry sample masses obtained on an analytical balance [6].

### 2.3. Analytical procedures

Soluble carbohydrate (CHO) was quantified by the phenol–sulfuric acid method with glucose as a standard [17]. The pH was measured on an Accumet AB15 pH meter (Fisher Scientific).

Surfactin and FA-Glu were quantified by LC/MS. Fermentation samples containing the surfactants were clarified by centrifugation followed by syringe filtration through 0.22 μm filters, and frozen until analyzed. LC/MS analyses were performed on a Thermo Scientific Accela UHPLC system coupled to a Thermo Scientific LXQ ion trap mass spectrometer with an ESI probe as described in related publication [6]. Gradient elution with mobile phases of deionized water and acetonitrile, both with 1% (v/v) acetic acid, was carried out using a Thermo Scientific C18 Hypersil Gold column (50 × 2.1 mm, particle size 1.9 μm) at 25 °C and flow rate of 0.6 mL $min^{-1}$ for the chromatographic separation of the biosurfactant variants. The biosurfactants were detected by the mass spectrometer in the negative ion mode and the data were processed using Xcalibur 2.0.7 software (Thermo Fisher Scientific, Waltham, MA). All samples were appropriately diluted so that the resulting surfactant concentrations were within the range of the calibration curves. Potassium myristoyl glutamate (KMG; Ajinomoto North America, Fort Lee, NJ) served as an internal standard and was added at 10 ppm to all samples and surfactant standards prior to injection. We followed published reference in quantifying the amounts of biosurfactants and were not looking to confirm structural conformations.

### 2.4. Physico-chemical properties of surfactants

#### 2.4.1. Surface tension and critical Micelle concentration

The surface tension was measured at 25 ± 1 °C using the Wilhelmy vertical plate technique with a sandblasted platinum plate as the sensor [18]. The pull exerted on the sensor was determined with a Cahn microbalance kept in a draft-free enclosure. For each measurement, the sensor was in contact with the solution for 30 min for equilibration. The CMC was obtained from the intersection of the sloped and plateau portions of the surface tension vs. concentration curve. The slope of the curve was used to calculate the adsorption density at the interface with the Gibbs equation [19]. The CMC values of surfactin and FA-Glu were measured in deionized double-distilled water (DDDW) and in 12 g/L NaCl solutions prepared using Instant Ocean® salt [20]. While FA-Glu was readily soluble in 12 ppt Instant Ocean® surfactin was found to precipitate and required solubilization. To dissolve surfactin, one part phosphate buffer with 8 g/L of NaCl was added to two parts Instant Ocean® solution (14 g/L) to obtain a surfactin solution having salinity of ~12 ppt.

#### 2.4.2. Dispersant-to-oil ratio

Dispersion effectiveness of biosurfactants was assessed by determining the dispersant-to-oil ratio (DOR) using a method similar to that reported earlier [21]. For determination of DOR, amount of oil (hexane) dispersed was accounted for per gram of surfactant. Above CMC value, ratio of oil dispersed per gram of surfactant would typically exhibit a linear relationship giving a constant DOR value. The relationship can be generally accounted as "$n$ × CMC concentration/oil volume = $n$ × DOR value". To test this relationship and obtain a consistent DOR value, a series of surfactant solutions was prepared at pH 7.5 and concentrations above its CMCs. Hexane was used as a model system, instead of crude oil that could vary regionally, to correlate the interfacial properties of the biosurfactants and COREXIT, the commercial dispersant. Using crude oil would be unreasonable for its possible variability to develop a fundamental comparative basis between them; thus, hexane was utilized for this comparison. Hexane was added drop-wise (10 μL) to 100 mL of the dispersant solution until hexane was observed as a separate phase. Upon addition of hexane, the dispersant solution was mixed for 1–2 min, allowing sufficient time for dispersion of hexane. After mixing, the solution was allowed to stabilize for 2–3 min. An oil-soluble red dye (Sudan III, Sigma–Aldrich) was used to more easily visualize phase separation. The amount of hexane added was noted and DOR values were calculated as grams of hexane dispersed per gram of dispersant. The DOR values are reported as the average of three replicates of 1:Y, indicating Y g of hexane dispersed per 1 g of dispersant.

### 2.5. Toxicity measurements

The toxicities of surfactin, FA-Glu, and sodium lauryl sulfate (SLS), a commercially available synthetic surfactant, were determined using 7–8 d-old larval Gulf killifish, *Fundulus grandis*, which is widely distributed in river, estuarine, and coastal marshes of the Gulf of Mexico [22] and grows well in a wide range of salinity [23,24]. These fishes are abundant in estuarine habitats and are one of a minority of species that utilize emergent marshland after flooding [25]. Rapid growth at this age enables detection of effects that are less evident in older animals. The effect of salinity on the toxicity of biosurfactants was also tested. From the work at LSU with surfactants, it appears that surfactants alter the fish's ability to regulate internal ion balance, thus imparting toxicity.

#### 2.5.1. Fish preparation

*F. grandis* were offspring of adults cultured at the Louisiana State University Agricultural Center (LSU Ag Center) from eggs collected periodically using a spawning mat [26]. The resulting larvae were cultured at 24–26 °C in 250-L flow-through tanks until 7–8 days post-hatch with daily feeding of commercial diets (35% protein, 6% lipid). Water was maintained at 12 ppt saline (approximately 1/3rd that of sea water), which is common in estuarine habitats. For tests conducted at salinities other than 12 ppt, salinity was changed by 4 ppt/d until reaching the desired salinity.

#### 2.5.2. Toxicity comparison

Surfactin and FA-Glu (both at purities of >95%) were obtained from fermentations carried out at Modular Genetics or Iowa State University; SLS was obtained from Sigma–Aldrich and tested as a reference surfactant. Test solutions were made in deionized water containing 12 ppt Instant Ocean® synthetic sea salts and, in the case of surfactin, supplemented with Tris–HCl as needed to prevent precipitation. Tris–HCl buffer alone was not toxic to the fish (data not shown). Preliminary wide-range tests were used to select >5 narrow-range concentrations for each biosurfactant. Availability of 7–8 d-old fish precluded testing all substances simultaneously, but controls were conducted simultaneously with all tests. Fish ($n$ = 20) were exposed to biosurfactants for 96 h in 24-well culture plates with each individual in 2 mL of test solution at 20–23 °C. Throughout the 96 h acute assay periods, fish were not fed and all solutions were replaced daily. Each treatment concentration and control was tested in triplicate for a total of 60 test organisms. Survival was recorded daily, with tests coinciding with control survival <90% excluded. Fish that were unresponsive to prodding were examined under a dissecting scope to verify death. Surviving fish were euthanized after experiments.

#### 2.5.3. Effect of salinity on toxicity

Toxicity of the three surfactants (Section 2.5.2) was compared across five salinities: 5, 10, 15, 20 and 25 ppt. Salinity was changed up to 5 ppt/d beginning 4 days after hatch so that fish would be ready for testing 8 days post-hatch. The surfactant concentration for the salinity tests was at the level required for killing 50% of the fish (LC50) by 96 h when salinity was at 12 ppt. The LC50 for surfactin,

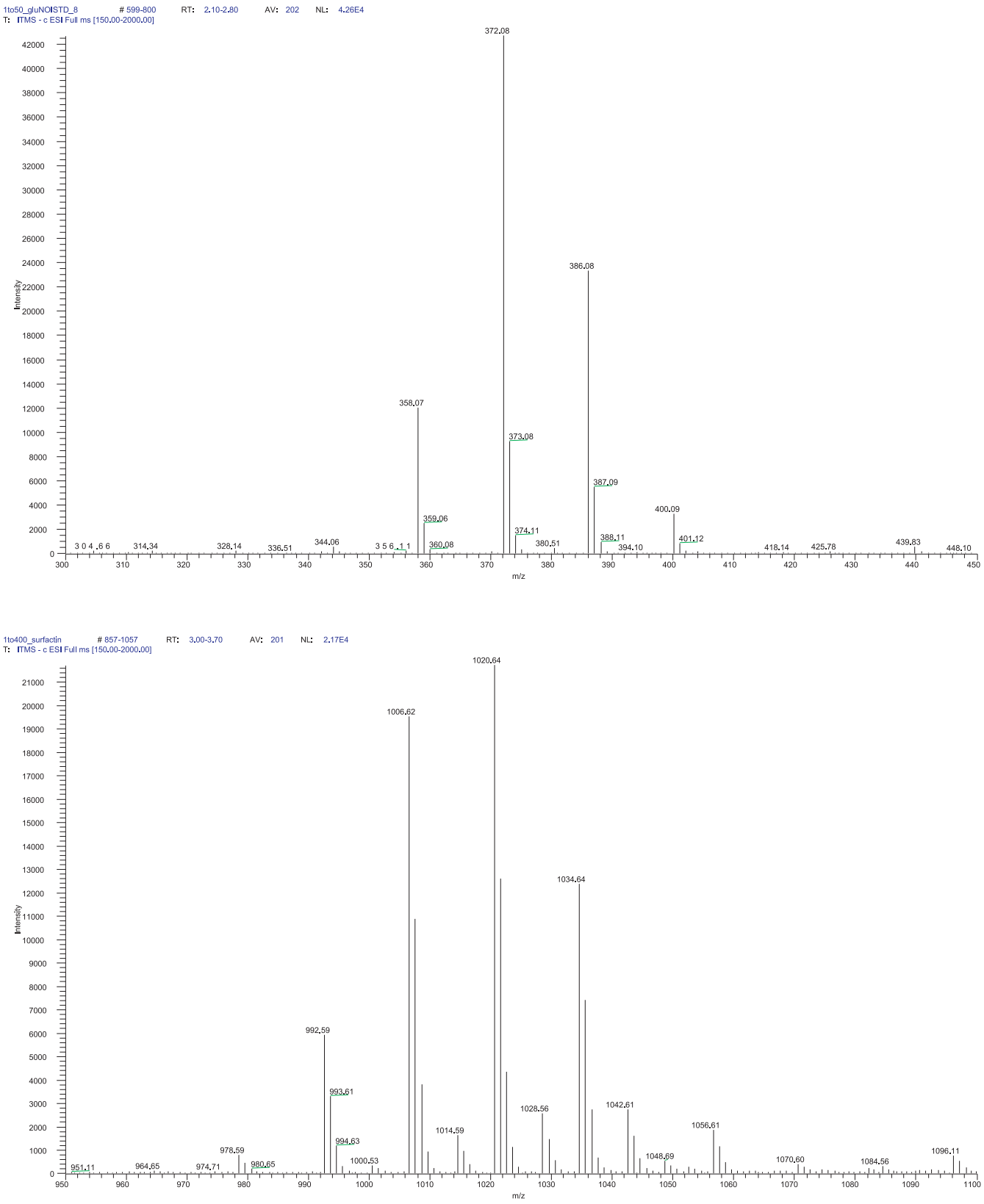


**Fig. 2.** Mass spectra of FA-Glu and surfactin: (a) Isoforms of FA-Glu can be seen at *m*/*z*'s 358.07, 372.08, 386.08 and 400.09. The isoforms with *m*/*z* 344.07 and 414.09 are the least abundant and often produce very low detector responses. (b) Isoforms of surfactin are at *m*/*z* 978.59, 992.59, 1006.62, 1020.64, 1034.64 and 1048.69. Specifics for LC/MS are described in Section 2.

**Table 1**
Surfactin and FA-Glu fermentation statistics: shake flask vs. bioreactor.

| Biosurfactant | Vessel type | Max A650 | Cultivation time (h) | Initial growth rate, ($h^{-1}$) | Final conc. (mg/L)[b] | (Adj. final conc.) (mg/L)[c] |
|---|---|---|---|---|---|---|
| Surfactin | Shake flask | 5.19 ± 0.43[a] | 72 | 0.34 ± 0.02 | 6200 ± 600 | |
| | 5-L bioreactor | 4.83, 6.03 | 33 | 0.38, 0.34 | 6.6 | (2595, 2557) |
| FA-Glu | Shake flask | 3.25 ± 0.60[a] | 72 | 0.39 | 210.5 ± 60 | |
| | 5-L bioreactor | 4.82, 5.05 | 40 | 0.46, 0.49 | 48.6 | (120, 142) |
| | 15-L bioreactor | 5.22 | 31 | 0.37 | 31.2 | (160) |

[a] Value following ± is the 95% confidence interval for all the means when $N > 2$. When $N = 2$, absolute values from runs are provided, for example, 2595, and 2557 are adjusted final concentrations, mg/mL, of surfactin from two 5-L bioreactor runs.
[b] Surfactants from aerated bioreactors were determined by analyses of the residual fermentation broth in the bioreactor. On the other hand, surfactant concentrations in shake flask were measured by analysis of unfractionated growth medium.
[c] Adjusted final concentration at end of cultivation was obtained by dividing the total surfactant in collapsed foam + and residual bioreactor broth with the initial bioreactor volume.

FA-Glu, and SLS was found to be 2.5, 25, and 10 ppm, respectively. Survival was determined in triplicate tests and controls. Toxicity tests lasted only 24 h as 96 h tests were unnecessary. Surviving fish were euthanized after experiments; as in this work we just developed dose–response curves, no post-euthanization work on fish was carried out.

The LC50 was estimated by the Trimmed Spearman Karber method [27] using at least five concentrations. Two-way ANOVA (Proc Mixed, $\alpha = 0.05$, Statistical Analysis System software, V9.2, SAS Institute, Inc.) was used to test for an interaction between salinity and substance on toxicity. There were three replicates for each test but six replicates for controls.

## 3. Results

### 3.1. Biosurfactant production profiles

Representative mass spectra of FA-Glu and surfactin are shown in Fig. 2. FA-Glu isoforms were detected over the retention time range of 2.1–2.8 min with $m/z$ 344.07, 358.07, 372.08, 386.08, 400.09 and 414.09. Isoforms of surfactin were detected over the retention time range of 3.0–3.7 min with $m/z$ 978.59, 992.59, 1006.62, 1020.64, 1034.64 and 1048.69. Fig. 3 shows representative production profiles for FA-Glu by *B. subtilis* 40688-E4 in S-7 medium in shake flasks (Fig. 3a) and 5-L bioreactors (Fig. 3b). The production profiles for surfactin (not shown) were very similar to those for FA-Glu. In either type of growth vessel (bioreactor or shake flask), cell growth and FA-Glu production peaked after 24 h corresponding to the time when glucose was almost completely consumed. The pH (initially @ 7.5) remained nearly constant during the cultivation (data not shown). The pertinent data from all cultivation runs are summarized in Table 1 with the number the replicates ranging from 2 to 8.

### 3.2. Biosurfactant titers comparison: FA-Glu vs. surfactin

While the data are comparable for the various fermentation vessels, marked differences were observed in titers for FA-Glu and surfactin. Typical surfactant titers in shake flasks ranged between 137 and 388 mg/L for FA-Glu; however, they were nearly ~30-fold higher for surfactin. This is consistent with the observations of Reznik et al. [6]. The enzymes responsible for FA-Glu synthesis are the result of genetic engineering. Although not universally true, reduced productivity by genetically engineered peptide- and polyketide synthases has been reported in the literature [28–30]. Final concentrations of both surfactants were lower in residual broth of larger fermentation vessels, as most of the surfactant was removed with the foam and concentrated in this fraction. The surfactant removed as foam was accounted for in the adjusted final concentrations (Table 1).

### 3.3. Initial growth rates of surfactant-producing B. subtilis strains

Initial growth rates for surfactin-producing cells were lower than those for FA-Glu producing cells (Table 1). The former converted more glucose into surfactant, and in so doing, consumed more ATP than strain 40688-E4. As such, fewer nutrients are available for growth. Surfactant productivity (i.e., g surfactant produced/g glucose consumed) was approximately 2.3 times higher in shake flasks than in the larger bioreactors (data not shown) due to possible removal of cells were along with the foam fraction during cultivation. These issues will need to be addressed further if the economics of microbial production of surfactant is to be favorable.

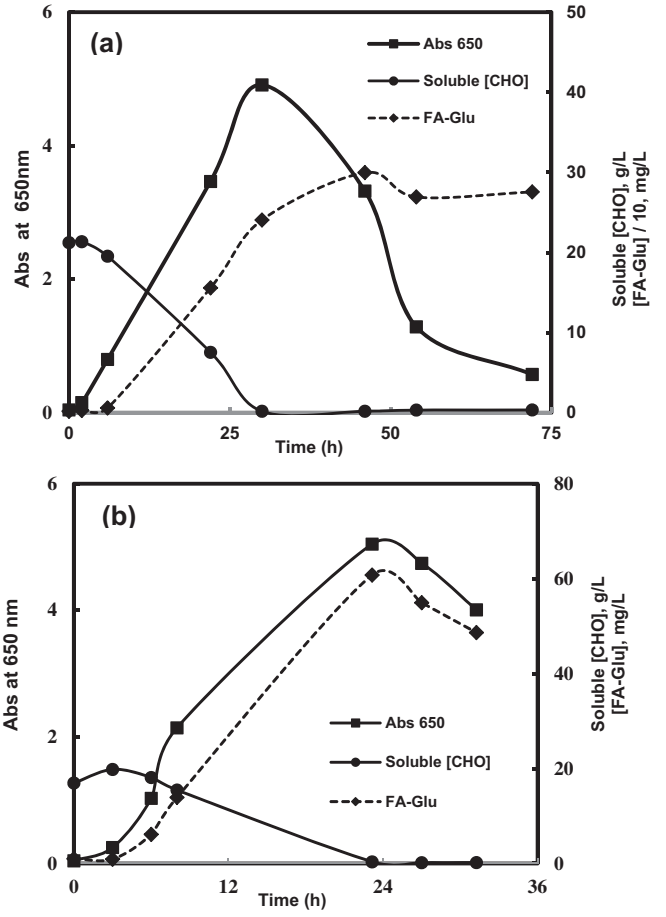


**Fig. 3.** Typical fermentation profiles for production of FA-Glu by *Bacillus subtilis* 40688-E4 in (a) shake flasks and (b) 5-L bioreactor. FA-Glu-enriched foam produced by aeration of the bioreactor (b) was continuously removed by overflow. *Note*: In Fig. 3a, the values for FA-Glu (mg/L) are divided by 10, so actual values are obtained by multiplying by 10. For example, FA-Glu (mg/L) at 72 h is about 280.

### 3.4. Critical Micelle concentrations of biosurfactants

The CMCs of surfactin and FA-Glu in DDDW were 0.015- and 0.10-g/L, respectively (Table 2), and were comparable to those of synthetic surfactants commonly used for oil dispersion, e.g., COREXIT 9500 (CMC 0.5 g/L, [31]). Surprisingly, CMCs were higher (0.02 and 0.4 g/L for surfactin and FA-Glu, respectively) in 12 ppt saline. Typically, CMCs of surfactants are lower in saline [32], increasing its efficiency as a dispersant for usage in oil spill, as the charge density of the head group is minimized by the solvated ionic species. Taking into account that the DOR values of biosurfactants studied here, particularly for surfactin, are comparable to that of DOR value of a commercial surfactant COREXIT, it certainly provides scope for its use in oil spill, even though with higher CMCs values. The observed higher values may be attributed to possible saline-induced conformational changes in the peptide moiety of surfactin. It has been reported that surfactin undergoes a conformational change from linear to either α-helical (below CMC) or β-sheet (above CMC) depending on concentration [33,34]. However, such conformational changes under saline conditions could be one of many possible reasons for rise in CMC values which needs further confirmation. The effects of electrolyte and pH have also been correlated with changes in surfactin and lipopeptide conformations [34,35]. Furthermore, spectroscopic studies suggest a structural variability depending on the nature of the solvent and cation concentrations [36].

### 3.5. Biosurfactant dispersant-to-oil ratios

The dispersant-to-oil ratios in DDDW and saline for the surfactants, FA-Glu and surfactin are also shown in Table 2. The DOR values for surfactin in water and artificial seawater (12 ppt saline) were 1:96 and 1:30, respectively, meaning that 96 and 30 parts of hexane are dispersed in aqueous solutions containing 1 part of surfactin and FA-Glu, respectively. Considering that the CMC values for surfactin were similar in water and saline, it was expected that surfactin's effectiveness in oil dispersion would likewise be similar in these solutions. Instead, surfactin's DOR reduced approximately 3-fold due to saline. For this study, since the dispersibility is explained in terms of DOR and the DOR values for surfactin under saline conditions obtained was 1:30, which is comparable to COREXIT ranging from ~1:10 to 1:100. This reduction in DOR can still be counted as desirable and it may be attributable to the effects of saline on emulsion stability, flocculation, creaming, coalescence and micelle size and shape. The DOR for FA-Glu also reduced ~3-fold in saline from 1:12 in DDDW to 1:4 in saline; this is consistent with the saline effect on FA-Glu's CMC. The data show that both biosurfactants are effective as oil dispersants and, as such, have the potential to replace commercial synthetic surfactants. Fundamentally, such variations in CMC values can be attributed to packing of surfactants and self-assembly forces that lead to the formation of micelles. However, irrespective of the CMC values, the DOR data show that both biosurfactants are effective as oil dispersants and, as such, have the potential to replace commercial synthetic surfactants.

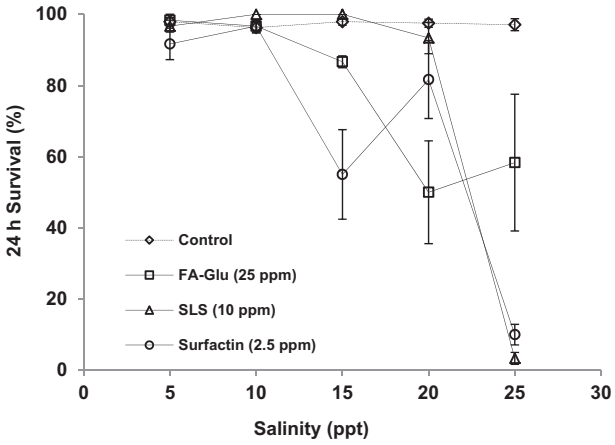


**Fig. 4.** Effect of salinity on the toxicity of surfactin, SLS, and FA-Glu. The concentration of each surfactant was set at 96 h-LC50, the amount required to kill 50% of the fish within 96 h, at a salinity of 12 parts per thousand (ppt). Control is without surfactant. Means and standard errors of the mean are presented for each treatment substance and control group.

### 3.6. Toxicity performance

At a salinity of 12 ppt, the mean 96 h-LC50s (±95% confidence interval) of surfactin, SLS and FA-Glu were 2.5 ± 0.19, 12 ± 2.78 and 25 ± 0.95 ppm, respectively, indicating surfactin to be the most toxic, while FA-Glu to be the least toxic among the substances studied. When the concentrations of these substances were held constant at concentration for 96 h-LC50 at 12 ppt saline, raising the salinity increased the toxicity of all three surfactants (Fig. 4); however, the increase was smallest for FA-Glu. The salinity interaction suggested that FA-Glu interfered with osmoregulation in a different way, and to a lesser extent, than the other surfactants.

## 4. Discussion

Biosurfactant yields were higher in shake flasks than in bioreactors. Surfactant synthesis slowed by ~24 h when glucose consumption was complete. Cells that produced FA-Glu grew faster than those producing surfactin; however, biosurfactant titers of the latter were ~30-fold higher than those for FA-Glu. When the bacteria were grown in large bioreactors, most of the both biosurfactants was recovered in the foam fraction. While biosurfactant enrichment via foam collection provides a possible economical recovery step, FA-Glu titers will need to be improved further with genetic modifications. This route seems likely to be more beneficial in improving titers than optimization of fermentation conditions.

The CMC of surfactin is 15 mg/L (Table 2). Surfactins from other species of *Bacillus* have similar CMCs. For example, surfactins with C14- and C15-fatty acid chains were reported to have CMCs of 31.3 and 21.0 mg/L, respectively [14]. The surfactin investigated in this study was a mixture of isoforms with a range of fatty acid chain lengths from 12 to 17 carbons. Comparison of our data with

**Table 2**
Critical micelle concentration (CMC), surface tension ( ) and dispersant-to-oil ratio (DOR) values of surfactin and FA-Glu in deionized double distilled water (DDDW) and saline at pH 7.5 saline solutions (12 ppt) were prepared using Instant Ocean®.

| Test material | CMC (g/L)[a] | | (mN/m) | | DOR[b] | |
|---|---|---|---|---|---|---|
| | DDDW | Instant Ocean® | DDDW | Instant Ocean® | DDDW | Instant Ocean® |
| Surfactin | 0.015 | 0.02 | 27.4 | 24.2 | 1:96 | 1:30 |
| FA-Glu | 0.1 | 0.4 | 27.2 | 31.2 | 1:12 | 1:4 |

[a] Range of replicates for CMC was ±1%.
[b] Range of replicates for DOR as ±5%.

literature suggests that the CMC of surfactin may vary inversely with the number of carbons in the fatty acid chain.

The CMC of FA-Glu was found to be 100 mg/L, nearly 7-fold higher than that of surfactin. The latter has a cyclic heptapeptide esterified to its fatty acid, whereas FA-Glu has only a single glutamic acid residue. The loss of the heptapeptide and lactone ring significantly impacts the micelle-forming ability of FA-Glu. A similar phenomenon was observed by de Faria et al. who isolated a surfactin variant from *B. subtilis* LSFM-05 grown on biodiesel glycerin [13]. This variant, which had a C14 fatty acid chain and a Glu-O-Me residue in its heptapeptide, had a CMC of 72.5 mg/L. Methylation of the glutamic acid increased the surfactin's CMC nearly 5-fold, indicating that modification of the peptide moiety has a significant effect on micelle formation.

The surface tension ( ) of surfactin and FA-Glu was found to be 27.4 and 27.2 mN/m, respectively. These are very similar to those of nC14- and ante-iso C15 surfactins (25.7 and 27.0 mN/m, respectively, isolated from *B. velezensis* H3 [14]. Although the absence of the heptapeptide influences the CMC of FA-Glu, it apparently had little effect on its surface tension compared to surfactin.

The CMC and DOR values for surfactin and FA-Glu in saline make them potential candidates as dispersants for oil-spill remediation. It was noteworthy that the CMC values of the biosurfactants in saline were higher than in deionized water. The $Na^+/Ca^{2+}$ ions in seawater [37] contribute to the surface activity of surfactins. The conformational change in surfactin, reported to depend on the $Ca^{2+}$: peptide concentration ratio [35,38] is likely due to $Ca^{2+}$ binding to amide groups. Among the surfactants tested, surfactin was the most effective dispersant of hexane, particularly in saline. Surfactin was also more toxic to fish than FA-Glu at higher concentrations. However, it may be possible to blend the biosurfactants to obtain preparations with high dispersant activity and reduced toxicity. This possibility needs to be investigated.

Salinity affects the toxicity of many compounds to aquatic organisms and has been the focus of a number of studies with consideration to bioassay testing [39]. Marine teleosts such as the silverside minnow (*Menidia beryllina*) and sheepshead minnow (*Cyprinodon variegatus*) exhibit different responses to changes in salinity, which could indicate altered susceptibility to toxicants under a range of test salinities [40]. Pillard et al. (1999) recommended that screening of specific ions could be valuable in conducting ion-related toxicity models for determining whole effluent toxicity tests for species such as the silverside minnow, which exhibit greater sensitivity to changes in ion composition vs. other euryhaline teleost species.

In the study reported, the three surfactants showed a progressive increase in toxicity when tested over a range of salinities. Other investigators have also demonstrated the toxicity of surfactants against fish as a function of salinity. For example, alkyl benzene sulfonates were shown to be toxic to mummichogs (*F. herteroclitus*) and the American eel (*Anguilla rostrata*) [41]. This may be due in part to differential intake of toxic material mediated by osmotic pressure [42]. Previous research on intestinal membranes has shown that dioctyl sodium sulfosuccinate (DSS) increases tissue conductance and sodium secretion, and decreases chloride absorption in the rat cecum [43]. It also completely inhibits soluble phosphodiesterase activity in human colonic mucosa at concentrations of 0.1 mmol/L and above, indicating a potent inhibitory action for this anionic surfactant [44]. Similarly, significant reductions in $Na^+/K^+$ ATPase activity have been observed within the gills of brown trout (*Salmo trutta*) through the inhibition of phosphodiesterase by specific chemicals [45].

Chemical dispersants alter the properties of oil by their emulsification properties, however, salinity appears to impact both the toxicity of surfactants and uptake of polycyclic aromatic hydrocarbons (PAH). Traditional anionic surfactants used in oil spill remediation are also used as strong denaturants for protein structures due to their properties as solubilizing agents for membrane-bound proteins and lipids [46,47]. The ability for fish to absorb PAHs is linked to changes in salinity, as the gill epithelium serves as a major site of ion and osmoregulatory balance. Duarte et al. demonstrated the effects of the chemical dispersant Corexit 9500, the crude oil and chemically dispersed crude oil on the osmotic function of the gills of the Tambaqui, *Colossoma macropomum*, by measuring the unidirectional flux of $Na^+$, $Cl^-$ and $K^+$ ions when individuals were held in freshwater conditions ($\leq$1‰). The largest degree of impairment was observed in the presence of the dispersant and chemically dispersed oil with a net efflux of $Na^+$ and $Cl^-$ and a decrease of $Na^+$ and $Cl^-$ concentration in the plasma within 6 and 12 h [48]. Lower salinity water (0‰) has been observed to significantly increase PAH uptake in the euryhaline tilapia (*Oreochromis mossambicus*) within the tissues of the gills, gonad, and liver relative to higher salinities of 15 and 30‰ [49]. As salinity decreases to estuarine conditions, PAH uptake potentially increases and the effectiveness of dispersants to weather oil decreases. The osmotic ability of Gulf killifish and probably respiratory exchanges in gills may be affected by the crude oil itself or by the anionic surfactant itself as suggested by Engelhardt et al. and Alkindi et al., but chemically dispersed oil may have an even greater impact on respiratory and the osmotic abilities [50,51]. Future bioassays with these types of surfactants with this euryhaline fish should account for alterations in osmoregulatory processes by monitoring possible alterations in ion transport protein function and localization at the gills.

Genetic engineering can be used to produce green chemicals with desirable functionalities for environmental applications. FA-Glu was found to be less toxic than synthetic surfactants; however, its oil-dispersing efficiency in saline is lower than that of surfactin. It would be reasonable to seek improvements by engineering additional modification to the peptide portion of the engineered surfactant and that is being pursued.

In summary, the studies presented in this paper are innovative in developing effective biodispersants with low environmental impact using green chemistry. This will also enhance the understanding and relationship between the structure and function of biodispersants and also lead toward selection and production strategies for highly effective biodispersants for oil spill cleanup uses.

## Conflict of interest

The authors declare that they have no conflict of interest.

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