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Surfactin from *Bacillus subtilis* displays promising characteristics as O/W-emulsifier for food formulations

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ABSTRACT

Background: Biosurfactants are surface-active molecules produced by different microorganisms and display a promising alternative to synthetically derived food emulsifiers. One of these biosurfactants, synthesized by *Bacillus subtilis*, is the lipopeptide surfactin, which composes a linear fatty acid and cyclic peptide moiety. This study explores the interfacial and emulsion forming properties of surfactin to further characterize its suitability as an O/W emulsifier in food formulations.

Results: Surfactin revealed a high interfacial activity with a reduction of interfacial tension of 83.26 % to 4.21 ± 0.11 mN/m. O/W emulsions (c_{oil} = 10 % *w/w*) were prepared by high-pressure homogenization, which yielded volume-based mean particle sizes below 1 µm already at low emulsifier concentrations of 0.01 % (*w/w*). Environmental stress experiments revealed that emulsions were stable between pH 6 to pH 9. Furthermore, neither phase separation nor extensive emulsion instability was observed with NaCl addition up to 0.5 M. However, $CaCl_2$ addition (> 3 mM) destabilized surfactin mediated emulsions. Finally, the main emulsion forming and stabilization effect of surfactin was related to its high interfacial activity and the high degree of electrostatic repulsion between the oil droplets (*i.e.* zeta-potential of up to −100 mV).

Conclusion: In comparison to other natural and synthetic emulsifiers, the results showed that surfactin is a strong candidate to form and stabilize O/W emulsions under the reported conditions.

1. Introduction

Biosurfactants are a broad group of surface-active molecules that can be synthesized by microorganisms as secondary metabolites, with many of them possessing promising surface-active properties relevant for food formulations [1,2]. Among the different biosurfactants, the lipopeptide surfactin produced by *Bacillus subtilis* is a potential candidate for food emulsions due to its high interfacial and surface activity [3].

Research on microbial surfactants, also termed biosurfactants, started in the 1960s. Biosurfactants are either secreted in the extracellular periphery or attached to the cell surface and their synthesis depends on environmental parameters such as pH, oxygen, and nutrient availability [4,5]. Up to date, the physiological role of biosurfactants is still not fully understood. Amongst others, their syntheses allow microorganisms to grow on water-insoluble substrates and consequently allowing for an easier uptake of these substrates for their own metabolism [5]. Biosurfactants gained attention for a variety of applications in different industrial sectors due to their high structural diversity and beneficial properties, such as production under controlled conditions, a broad spectrum of physicochemical properties, add on benefits such as antimicrobial activities, presumably low toxicity and a high biodegradability [5–7]. The food industry displays one of these promising industrial sectors. Here, the application of biosurfactants meets the trend of replacing chemically synthesized food additives, such as emulsifiers. In addition, renewable raw materials or waste-streams from the food industry were reported to serve as a feedstock for biosurfactant production (*e.g.* olive oil mill effluent, plant oil extracts, distillery, brewery and whey wastes, potato process effluent, and cassava waste), which fits well to foster the formation of a circular economy [5,8].

Currently, low molecular weight emulsifiers derived from natural and renewable sources, such as phospholipids and saponins, are already used in foods. While only a few saponins are commercialized in this

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field, phospholipids (*e.g.* lecithins from soybean) are incorporated in many foods as technofunctional ingredients. However, the functionality of phospholipids is insufficient in environmental conditions present in many foods. For example, phospholipids have a limited range of performance regarding stabilizing interfaces at high ionic strengths, extreme pH-values, or during temperature changes [9,10]. For these reasons, replacing frequently used natural surfactants with biosurfactants in foods might be beneficial in terms of enhanced and product-specific interfacial properties (*i.e.* emulsifying properties). Moreover, local and decentralized production with high purities may add to production resilience against market changes.

In general, biosurfactants can be categorized according to their chemical structure: glycolipids, lipopeptides, fatty acids/neutral lipids, phospholipids, and polymeric surfactants [2]. The lipopeptide surfactin synthesized by *Bacillus subtilis* is one of the most frequently studied and a promising candidate to be used in foods in the future [11]. Lipopeptides have an amphiphilic structure with an oligopeptide ring as hydrophilic, and a fatty acid chain as a hydrophobic group [11]. In case of surfactin, the oligopeptide ring comprises seven amino acids, while the fatty acid chain consists of 3-hydroxy fatty acids. An exemplary structure is given in Fig. 1.

More than 30 different surfactin congeners were reported up to date that differ either in the fatty acid residue (length and saturation) or the amino acid composition [12]. Up-to-date, surfactin from Kaneka Corporation (Osaka, Japan) has the approval to be used in cosmetics, and cosmetic products such as shampoo containing surfactin are already commercially available (Onlybio.life SP. Z O. O., Bydgoszcz, Poland). With respect to the food industry, the presence of surfactin in different fermented food products (*i.e.* Natto) can be considered as advantage for the approval. For instance, 2.2 mg/g of surfactin were reported to be present in Natto which equals 80–100 mg surfactin per 50 g of Natto [13]. Indeed, the ADI (acceptable daily intake) has to be evaluated, as well as further toxicological and sensorial studies must be performed. Nevertheless, due to the natural presence of surfactin in different fermented foods and the concomitant daily consumption by numerous humans, as well as the GRAS (generally accepted as safe) status of the production host *B. subtilis*, an implementation of surfactin in the food industry is very conceivable.

Nevertheless, although often declared as an outstanding surfactant, comprehensive studies targeting at the emulsifying characteristics of surfactin relevant for foods are underrepresented. Studies that have been published so far often used non-food grade materials or purification methods, focused on antimicrobial properties or antioxidative stabilities of the emulsion, or aimed at applications in *e.g.* enhanced oil recovery [14–17]. Although these studies claimed that surfactin might be an effective emulsifier, the emulsifying properties of surfactin relevant for applications in food formulations have not been well characterized yet. Therefore, this study aimed at characterizing the interfacial properties and investigating the emulsifying abilities of surfactin purified to a high degree from a *B. subtilis* culture broth under conditions typically found in foods.

2. Materials and methods

2.1. Chemicals and materials

Chemicals were of analytical grade and purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). The surfactin reference standard (≥ 98 % purity) was obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). The medium-chain triglyceride oil (MCT, Miglyol 812 N) used for interfacial measurements and for emulsion preparation was ordered at Cremer Oleo GmbH & Co. KG (Hamburg,

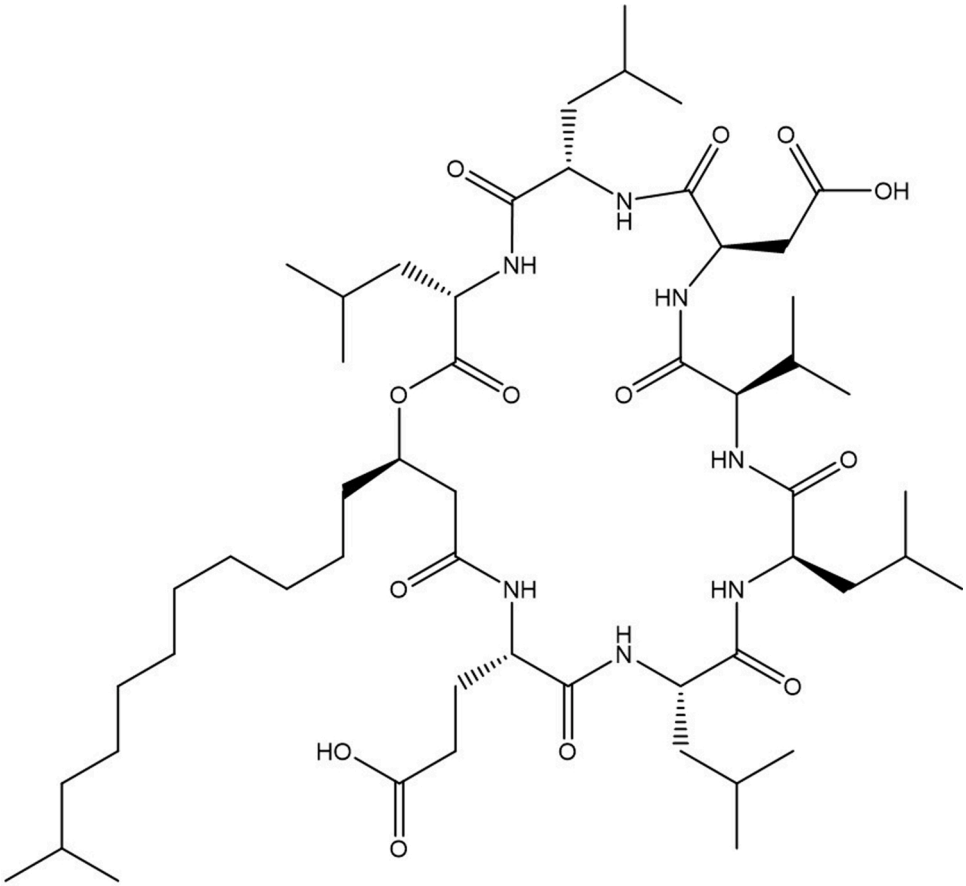

Fig. 1. Exemplary structure of the cyclic lipopeptide surfactin synthesized by *Bacillus subtilis*.

Germany).

2.2. Surfactin production, downstream processing and purity determination

Surfactin was purified from the culture broth of *B. subtilis* JABs32, which is the high-cell density strain 3NA [18] with functional *sfp* gene. The strain was cultivated in a 42 L custom-built bioreactor system (ZETA GmbH, Graz/Lieboch, Switzerland) under aerobic conditions at pH 7 and 37 °C in a defined mineral salt medium (12 L batch medium: 5.5 *g*/L glucose · H_2O, 4 *g*/L Na_2HPO_4, 14.6 *g*/L KH_2PO_4, 4.5 *g*/L $(NH_4)_2SO_4$, 0.2 *g*/L $MgSO_4$ · 7 H_2O and 3 mL/L trace element solution (TES); 2 L Feed I: 137.5 *g*/L glucose · H_2O; 0.2 *g*/L $MgSO_4$ · 7 H_2O; 6 L Feed II: 604.99 *g*/L glucose · H_2O; 12 *g*/L $MgSO_4$ · 7 H_2O, 120 mL/L TES; TES contained 40 mmol/L Na_3citrate, 5 mmol/L $CaCl_2$, 50 mmol/L $FeSO_4$ and 0.6 mmol/L $MnSO_4$ · H_2O). Surfactin was purified by a two-step ultrafiltration (UF) procedure based on the principle reported by Isa et al. [19]. Briefly, the culture broth was centrifuged at 4700 rpm for 15 min at 4 °C to remove cells and passed through a 10 kDa membrane (Sartocon® Slice Hydrosart® Cassette, 0.1 m^2, Sartorius AG, Göttingen, Germany) to collect surfactin micelles in the retentate. The broth was concentrated up to 30 % and subsequent step-wise addition of water resulted in further flushing out of salts. The final retentate was adjusted to pH 7 and diluted 1:10 (*v/v*) in 50 % methanol to disrupt the surfactin micelles. The obtained solution was subjected to another 10 kDa ultrafiltration, which results in surfactin molecules permeate through the membrane. The collected permeate was concentrated (Rotavapor R-215, Büchi Labortechnik AG, Flawil, Switzerland) at 60 °C and 200 rpm by gradually reducing the vapor pressure from 340 to 280 mbar. The remaining liquid was acidified to pH 3 adding 32 % HCl to precipitate surfactin and stored overnight at 4 °C. The precipitate was collected by centrifuging at 4700 rpm and 4 °C for 15 min (Heraeus X3R, Thermo Fisher Scientific GmbH, Braunschweig, Germany). Three washing steps were performed by resuspending the collected precipitate in acidified water at pH 3 and another centrifugation step at 4700 rpm and 4 °C for 15 min. The final washed surfactin was resolved in 5–15 mL water and the pH was neutralized by adding 4 M NaOH. Finally, surfactin was collected after freeze-drying in a rotary vacuum evaporator (RVC 2–25 Cdplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

The obtained surfactin was redissolved in methanol to achieve a concentration of 0.1 mg/L and purity was determined in comparison to a standard curve using a HPTLC method as described in Geissler et al. [20].

2.3. Aqueous emulsifier solution

Aqueous surfactin stock solution (6 g/L) was prepared by dispersing surfactin in a 10 mM sodium phosphate buffer at pH 7. This stock solution was diluted with buffer to appropriate concentrations for emulsion preparation, interfacial tension measurement and interfacial rheology.

2.4. Characterization of interfacial properties

2.4.1. Interfacial tension measurements

Interfacial tension at the oil-water-interface was determined with a drop shape analyzer (DSA 10, Krüss GmbH, Hamburg, Germany) after 300 s of equilibration time at 25 °C. Surfactin concentrations examined ranged from 0.1 mg/L to 1000 mg/L. The aqueous emulsifier solution at pH 7 was injected into the outer oil phase (MCT, Miglyol 812 N). Interfacial tension was determined as a function of the corresponding drop shape using the Young-Laplace equation. Pure sodium phosphate buffer without emulsifier had an interfacial tension of 25.16 ± 1.47 mN/m.

2.4.2. Hydrophilic-lipophilic balance

The hydrophilic-lipophilic balance (HLB-value) was calculated using the following formula:

$$\mathrm{HLB} = 20 \times \left(1 - \frac{M_l}{M}\right)$$

M_l (g/mol) is the molar mass of the lipophilic part and M the molar mass of surfactin (1036.34 g/mol).

2.4.3. Interfacial rheology at absorbed films

The structural properties of surfactin at the oil-water interface in sodium phosphate buffer (30 mg/L) were determined using an MCR 502 modular compact rheometer with a bicone Bi-C68−5 geometry with a diameter of 68.25 mm (Anton Paar Germany GmbH, Ostfildern, Germany). An amplitude sweep was carried out at a frequency of 0.3 rad/s and a strain ranging from 0.1%–100% at 25 °C after 1 h of incubation time. Therefore, the surfactin solution was filled into the measuring cup and after placing the bicone at the interface, oil was slowly poured on top of the solution.

2.5. Emulsion preparation

Pre-emulsions were prepared with a high-shear blender (Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) by blending 10 % (*w/w*) Miglyol oil with 90 % (*w/w*) aqueous emulsifier solution (5 mg/L – 5000 mg/L) at 20,000 rpm for 3 min for each emulsion. For easier detection of coalescence, Miglyol oil was colored with red capsicum extract (Gewürzmüller GmbH, Stuttgart, Germany). Pre-emulsions were passed through a high-pressure homogenizer (EmulsiFlex-C3, Avestin, Ottawa, Canada) for four cycles with a homogenization pressure of 500 bar, if not stated otherwise. In general, samples were analyzed directly after preparation, and after 24 h and 7 days of storage at 4 °C.

2.5.1. Influence of surfactin concentration

Emulsions with 90 % (*w/w*) of surfactin solution containing 5 mg/L to 5000 mg/L surfactin and 10 % (*w/w*) Miglyol oil were prepared to determine the influence of different surfactin concentrations on emulsion stability.

2.5.2. Influence of homogenization pressure

Emulsion samples with 0.5 % (*w/w*) surfactin were used to determine the influence of homogenization pressure. Emulsions were prepared using 500 bar, 750 bar and 1000 bar for four cycles.

2.6. Emulsion stability at different environmental conditions

2.6.1. Influence of pH

The impact of pH on emulsion samples containing 0.5 % (*w/w*) surfactin was determined in a range from pH 3−9. The pH was adjusted after emulsion preparation adding 0.1 and/or 1 M NaOH or HCl and emulsions were stored overnight at 4 °C and readjusted, if necessary.

2.6.2. Influence of ionic strength

Emulsion samples were adjusted to the specified ionic strength (0.01 – 0.5 M NaCl, 0.003 – 0.16 M $CaCl_2$) after emulsion preparation using ionic-strength adjusted buffer. The pH of ionic-strength buffer was adjusted to pH 7 by adding 0.1 and/or 1 M NaOH or HCl. The final surfactin concentration was set to 0.5 % (*w/w*).

2.7. Analysis of emulsions

Emulsion characterization included particle size distribution, optical microscopy, and photographic images. The zeta-potential of emulsion droplets was additionally measured for pH and ionic strength modified emulsions.

2.7.1. Particle size distribution

The particle size distribution of the emulsions was measured with a static laser diffraction particle analyzer (Horiba LA-950, Retsch Technology GmbH, Haan, Germany). The measurement cuvette was filled with 10 mM sodium phosphate buffer and the sample was injected in appropriate volumes to prevent multiple scattering effects (10 µL – 30 µL per 15 mL). The refractive index was set to 1.45 and 1.33 for emulsion (dispersed phase) and buffer (aqueous phase), respectively. Droplet sizes were reported as volume-based ($d_{4,3}$) mean droplet sizes. The software used for evaluation was HORIBA NextGen Project LA-950 (2010).

2.7.2. Optical microscopy

For microscopic images of emulsion droplets, an optical light microscope (Axio Scope.A1 with an ICc3-camera, Carl Zeiss Microimaging GmbH, Göttingen, Germany) equipped with 20x, 40x and 100x objectives was used. Appropriate scale bars were added with the software ImageJ [21].

2.7.3. Photographic images

For visualization of coalescence and phase separation, photographic images from emulsions were taken in a photo box.

2.7.4. Zeta-potential

A particle electrophoresis instrument (Nano ZS, Malvern Instruments, Malvern, UK) was used to calculate the zeta-potential at 25 °C using the droplet velocity in an applied electric field. The software used was Malvern Zetasizer Software 7.12 (2016). The samples were diluted with sodium phosphate buffer at an appropriate pH and ionic strength to ensure optimum measuring conditions.

2.8. Statistical analysis

All analyses were performed at least in duplicate with at least two freshly, independently prepared samples. Values reported represent means and standard deviations that were calculated using Excel (Microsoft, Redmond, WA).

3. Results and discussion

3.1. Surfactin purity

Surfactin was purified from the culture broth by two physical methods (filtration and centrifugation) and acid precipitation. These techniques are common in downstream processing and could be transferred to industrial scale. Purity determination by HPTLC revealed that the chromatograms of the purified surfactin were comparable to the standard surfactin peaks as reported in Geissler et al. [20]. The purity of freeze-dried surfactin obtained from this process was 84.76 ± 4.29 % and had a white powdery appearance, as expected. The high purity assured that other surface-active ingredients are only present in minor amounts, ensuring that the observed effects are related to surfactin, which was the aim of the study.

3.2. Interfacial tension measurements

Initially, the interfacial tension of purified surfactin was measured to gain basic insights about the interfacial activity and adsorption behavior at the oil/water-interface of surfactin, which is a prerequisite for emulsion formation. Fig. 2 displays the interfacial tension at increasing surfactin concentrations in sodium phosphate buffer at pH 7. With increasing concentration, a decrease in interfacial tension of ~82 % was achieved, showing that surfactin adsorbs at oil-water interfaces. The interfacial tension was reduced from 24.16 ± 1.50 mN/m using 0.1 mg/L surfactin to 4.57 ± 0.04 mN/m at a concentration of 30 mg/L surfactin and beyond. By lowering the interfacial tension, surfactin reduces the Laplace pressure of the formed droplets, thus facilitating a droplet breakup during emulsion formation [22,23].

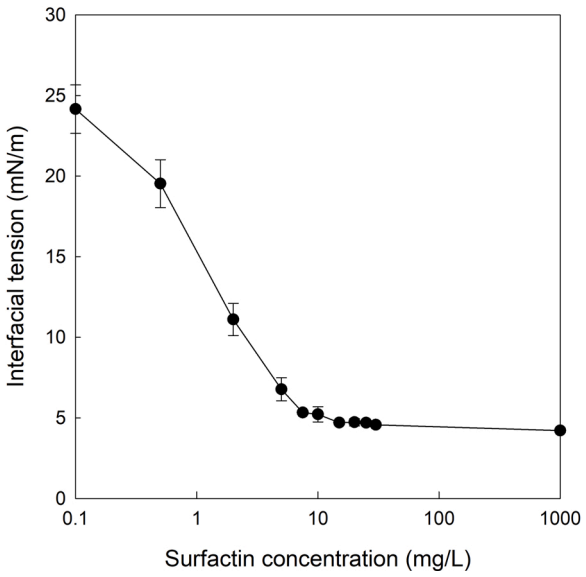

Fig. 2. Influence of surfactin concentration (mg/L) in an aqueous emulsifier solution at pH 7 on the interfacial tension at an oil-water-interface (mN/m).

The high binding affinity and fast adsorption of surfactin correspond to its low molecular weight and amphiphilicity, which is related to the hydrophilic-lipophilic balance (HLB). The HLB-value of surfactin was determined by the method of Griffin [24] and was calculated to be between 15.92 and 16.46, when considering β-hydroxy fatty acids with a chain length of 13–15 carbon atoms. Gudiña et al. [25] reported on a suggestive HLB-value of 10−12. Therefore, surfactin is mostly hydrophilic and is suitable to facilitate the formation of O/W emulsions, which is in accordance with the study design.

Prior studies have already investigated the interfacial properties of surfactin. Table 1 gives an overview of both the interfacial as well as surface tension and critical micelle concentration of surfactin and further selected surfactants that are commonly used in foods. Surface tension is an important value for foamed food products such as mousse and was included to provide a more coherent overview. The comparison may be somewhat limited by the use of a crude biosurfactant or cell-free supernatant, with unknown purities in many studies. In addition, measurement techniques and setting parameters, such as pH and temperature, varied amongst the studies. This makes a distinct comparison difficult.

However, considering the influence of the different approaches, the surfactin recovered during this study showed comparable interfacial values than both surfactin and other microbial biosurfactants described in previous studies. In contrast to earlier findings, the present study obtained this value at a lower surfactin concentration, which might be attributed to the different purities used, different strains and cultivation conditions (*e.g.* temperature, oxygen, media composition), and purification methods, resulting in a different surfactin congener pattern. In comparison to other natural and chemical surfactants, interfacial and surface tension values of biosurfactants are in general comparable or even lower as summarized in Table 1. It can, therefore, be assumed that surfactin is able to facilitate emulsion formation. Additionally, for surfactin and the other biosurfactants much lower amounts were needed to achieve these values which favors the use of surfactin in food formulations. For example, about 20,000 mg/L of Tween 80 and 50,000 mg/L Yucca saponin extract were used to reach an interfacial tension of 5 mN/m and 3.4 mN/m, respectively (Yang et al., 2013, [40]), while only 30 mg/L surfactin were needed to yield the lowest values of interfacial tension (Fig. 2).

Table 1
Comparison of interfacial tension, surface tension and critical micelle concentration of different surfactants with the respective surfactant concentration.

Surfactant	Interfacial tension γ_{OW} (mN/m) (Surfactant concentration (mg/L))	Surface tension γ_{AW} (mN/m) (Surfactant concentration (mg/L))	Critical micelle concentration (mg/L)	Reference
Microbial biosurfactants				
Surfactin	4.57 (30)	n.d.	n.d.	This study
	2.45 (100)		10	Deleu et al. [26]
	1 (crude)	27 (crude)	25	Cooper et al. [27]
		36 (>100)	15.6	Abdel-Mawgoud et al. [28]
		27 (15)	15	Long et al. [16]
	0.97 (cell-free supernatant)	26.6 (cell-free supernatant)	33	Nitschke and Pastore [29]
	<1 - 8 (10–100)	25–31 (10–100)	20–200	Syldatk et al. [30]
Rhamnolipids		33 (~250)	120	Radzuan et al. [31]
	0.7–10.27 (150)	27 (~100)	25.7	Mendes et al. [32]
Sophorolipids	0.99 - 4.46	34.18 (>30)	27.17	Daverey and Pakshirajan [33]
	4 (200)	36 (~100)	70	Ashby et al. [34]
Natural surfactants				
Gum Arabic	7 - 47	47 - 55	90 - 130	McClements and Gumus [23], Grein et al. [35]
ß-casein	19	50		McClements and Gumus [23]
Egg lecithin		22.36 (2700)	850	Pogorzelski et al. [36]
Lecithin	13 (100)			Torcello-Gómez et al. [37]
Sugar beet extract	14.5 (50,000)	48.3 (50,000)		Ralla et al. [38]
Quillaja saponin	4.2 (50,000) 5 (~20,000)	35.9 (50,000)	100	Ralla et al. [38],Yang et al. [39]
Yucca saponin extract	3.4 (50,000)	37.9 (50,000)	1000	Ralla et al. [40]
Red beet extract (Saponin)	16.3 (50,000)	28.6 (15,000)	5000	Ralla et al. [41]
Chemical surfactants				
Tween 20	2	40	~60	McClements and Gumus [23], Rehman et al. [42]
Tween 80	5 (~20,000)		30	Yang et al. [39]
Polysorbate 80		70 (2700)	100	Pogorzelski et al. [36]

3.3. Influence of surfactin concentration and homogenization pressure on emulsion formation

Based on the high interfacial activity, oil-in-water emulsions (pH 7, 10 % (*w/w*) Miglyol oil) were prepared with increasing surfactin concentration (5 mg/L – 5000 mg/L). The aim was to establish surfactin concentrations that form stable emulsions. Particle size distributions were measured directly after preparation (0 h) and after 24 h and 7 days of storage at 4 °C. The results are illustrated in Fig. 3A.

With increasing surfactin concentration, the mean particle size measured directly after preparation decreased from 2.46 ± 0.11 µm with 5 mg/L surfactin to less than 1 µm employing concentrations of more than 100 mg/L. The lowest mean particle size with 0.39 ± 0.06 µm was achieved with 5000 mg/L surfactin. The stability of the surfactin stabilized emulsions during 7 days of storage revealed that the particle sizes did not change significantly ($p < 0.05$). With respect to the volume-based mean particle size distribution after preparation (Fig. A1 in appendix), all surfactin concentrations showed a monomodal behavior with the exception of 500 mg/L having a slight bimodal distribution, and the peak was shifted towards smaller particle sizes the more surfactin was used as emulsifier. However, although microscopical analyses supported these data, the visual evaluation of emulsions revealed the presence of coalescence and subsequent gravitational separation processes up to 1000 mg/L of surfactin that were not detected by the light scattering measurements (Fig. 3B). This effect is most likely related to an insufficient concentration of emulsifier present to fully cover the created interface at the given energy input (*i.e.* homogenization pressure). While a slight oily shimmer was visible on the surface of emulsions employing

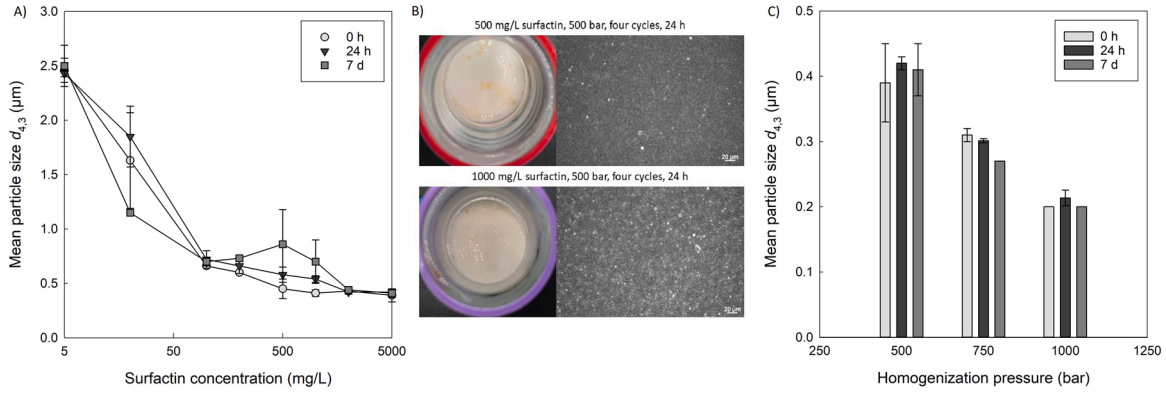

Fig. 3. Influence of different surfactin concentrations (mg/L) on the volume-based mean particle size ($d_{4,3}$) (µm) of O/W emulsions (10 % (*w/w*) Miglyol oil, pH 7, 500 bar, four cycles) (A), visual and microscopical evaluation of emulsions with 500 mg/L and 1000 mg/L surfactin (10 % (*w/w*) Miglyol oil, pH 7, 500 bar, four cycles) (B) and impact of different homogenization pressures (bar) (each four cycles) on the volume-based mean particle size ($d_{4,3}$) (µm) of O/W emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, pH 7) (C).

2 g/L, neither coalescence nor oily shimmer were visible when 5 g/L surfactin was used (data not shown). Consequently, this concentration was used for all subsequent experiments.

Even though data on emulsifying properties of surfactin are still limited, these results seem to be consistent with other research, which found a release of the oil phase hexadecane of 25.8 % in an emulsion prepared with 0.1 mg/L surfactin, also indicating that this concentration as a sole emulsifier is not sufficient to obtain stable emulsions [26]. However, further studies indicated that by using much higher surfactin concentrations (3%, *v/v*) with the addition of solvent yields emulsions with even lower mean droplet sizes (72.52 nm) using sunflower oil in an emulsion composed of 20 % (*v/v*) oil phase (14 % oil of total emulsion, 3% ethanol) and 80 % (*v/v*) water phase [15].

To gain more insights into the emulsifying properties of surfactin, the influence of homogenization pressure on O/W emulsions (10 % (*w/w*) Miglyol oil, pH 7) with 5 g/L surfactin as emulsifier was further examined (Fig. 3C). Pressures above 500 bar are most often used in food processing to prepare delivery systems, such as for essential oils [43,44]. In general, an increase in homogenization pressure led to a decrease in volume-based mean particle size. Accordingly, emulsions with 500 bar, 750 bar and 1000 bar showed mean particle sizes of 0.39 ± 0.06 µm, 0.31 ± 0.01 µm and 0.20 ± 0.00 µm, respectively. After a storage time of 7 days at 4 °C, emulsions prepared with 500 bar showed a slight increase to 0.41 ± 0.04 µm, and at 750 bar a slight decrease to 0.27 ± 0.00 µm was observed, whereas emulsions with 1000 bar remained similar with 0.20 ± 0.00 µm. Consequently, no over-processing and thus no significant increase in particle size could be detected [45]. However, 500 bar was chosen for all further experiments since this energy input already resulted in stable emulsions.

Overall, in the current study, the results showed that the surfactant-to-oil ratio (*r*) of 0.5:10 is very low to obtain particle sizes of $d_{4,3} = 0.2$ µm (Fig. 3C) when applying appropriate homogenization pressures. In comparison to other surfactants, the amount to form a stable emulsion with particles of ~0.2 µm is comparably low. For example, the *r*-values for synthetic surfactants such as Tween 80 with $r \approx 0.5{:}10$ is similar and for natural Quillaja saponin extract with $r \approx 1{:}10$ and sugar beet with $r \approx 0.75{:}10$ slightly higher [38,39]. On the contrary, other natural emulsifiers such as gum arabic, lecithin or ß-casein have much higher *r*-values with 10:10, 2:10, and 1:10 [9,23].

3.4. Influence of pH on emulsion stability

Many foods undergo a shift in pH during processing and final food products have acidic pH values. To evaluate the emulsion stability against such changes, the pH-value of emulsions was adjusted between pH 3 and 9 after emulsion formation. According to previous reported studies investigating the stability of surfactin across different pH-values and the presence of the acidic residues aspartate and glutamate causing precipitation at low pH-values, it was expected that surfactin is not able to stabilize the oil droplets across the complete pH-range that is commonly found in foods. The volume-based mean particle sizes $d_{4,3}$ at various pH-values of emulsions (10 % (*w/w*) Miglyol oil, 5 g/L surfactin) prepared, and exemplary photographic and microscopic images, are given in Fig. 4A and B, respectively. Moreover, zeta-potential measurements are shown in Fig. 4C.

First, particle sizes of samples with pH 3 were not detectable due to complete emulsion breakdown, which was confirmed both visually and microscopically. Second, decreasing the pH below 7 down to pH 4 resulted in emulsions being mostly stable against gravitational separation, but emulsions exhibited coalescence and a pronounced increase in particle size. For example, the particle sizes of emulsions at pH 4 were 0.69 ± 0.10 µm and 0.65 ± 0.01 µm after 24 h and 7 days, and 0.30 ± 0.07 µm and 0.31 ± 0.02 µm at pH 7, respectively (also see appendix A2 for particle size distributions). Third, emulsions with pH 6 and beyond up to pH 9 were visually and microcopically stable and particle sizes remained below 0.4 µm during storage time.

To elucidate the stabilizing mechanism, zeta-potential and interfacial shear rheology measurements were carried out. Overall low values for the loss and storage modulus ($< 10^{-4}$ Pa) were obtained after 1 h and 8 h of incubation without shear and the values were in the same order of magnitude than water (data not shown). Thus, the interfacial moduli were not calculated and it can, therefore, be assumed that surfactin does not build up a strongly interlinked and cohesive interface that exhibits steric repulsion. The zeta-potentials of pH-adjusted emulsions are summarized in Fig. 4C. All emulsion samples had a negative zeta-potential probably caused by carboxylic groups present in the peptide structure. With increasing pH-value, a decrease in zeta-potential and therefore a stronger negative charge was detected, which is in accordance with the increased droplet stabilization at higher pH-values due to an increase in deprotonated carboxyl groups. With the lowest pH-value of pH 3, a zeta-potential of -13.53 ± 0.26 mV after 7 days was measured. Emulsions with neutral to alkaline conditions showed zeta-potentials of more than −100 mV. The highest value of -108.58 ± 0.25 mV was obtained at pH 9. For comparison, lecithin had a maximum negative charge with around −60 mV at pH 7 and -35 mV at pH 3 [23], while Tween 80 only resulted in a zeta-potential of −9 mV at pH 9 and of +2 mV at pH 2 [39]. Natural *Quillaja* saponin extract and sugar beet extract reached lower zeta-potentials with increasing pH-values with the lowest zeta-potential of -65.5 mV and -54.2 mV at pH 9 [46]. An implication of these findings is that surfactin emulsions are mainly stabilized by electrostatic interactions, and thus droplet destabilization is observed by changing the pH-value.

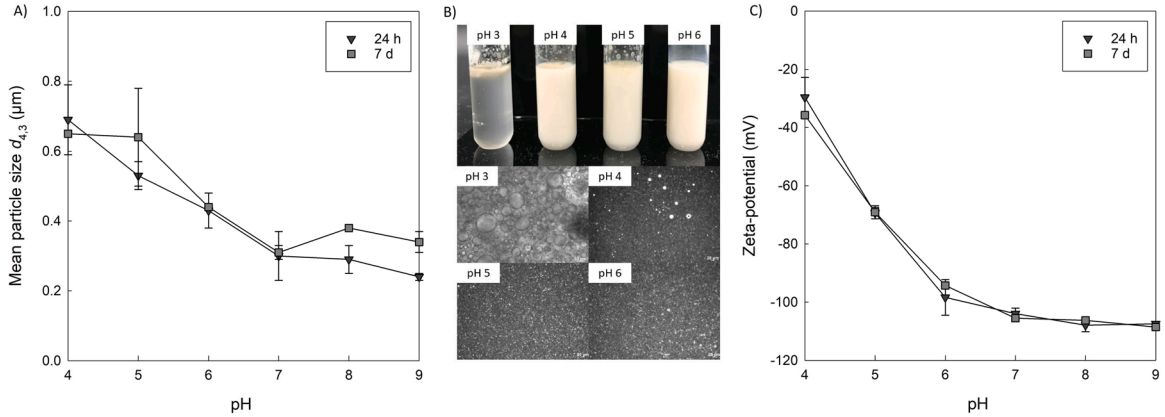

Fig. 4. Influence of different pH-values on the volume-based mean particle size ($d_{4,3}$) (µm) of O/W emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, 500 bar, four cycles) (A), exemplary visual evaluation and microstructures of emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, 500 bar, four cycles) in the range from pH 3 to pH 6 (B) and impact of different pH-values on the zeta-potential (mV) of O/W emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, 500 bar, four cycles) after 24 h (C).

The reported results are consistent with solubility measurements of isolated surfactin. Abdel-Mawgoud et al. [28] investigated the water-solubility of surfactin at pH-values ranging from 5 to 13. The highest solubility was obtained at pH 8.0–8.5, while the lowest solubility was at pH 5, indicating an isoelectric point around pH 5 [47]. Moreover, Long et al. [16] reported as well on an improved emulsification activity at higher pH-values, while precipitation occurred at pH 3. In addition, Abdel-Mawgoud et al. [28] reported on an increase in surface tension in the pH range from 6 to 13, whereas a surface tension reduction at pH-values of 2–4 was not measurable, indicating a total loss in amphiphilicity and, consequently, desorption from the interface and emulsion destabilization. However, it was shown that surfactin is soluble in organic solvents like ethanol, acetone, methanol, butanol, chloroform and dichloromethane [28]. Such behavior could be interesting for food or pharmaceutical products based on alcoholic continuous phases like liquors or syrup.

3.5. Influence of ionic strength on emulsion stability

Various emulsified foods have a high ionic strength due to the natural presence of salts in foods such as sports- and wellness-drinks, sausages and dairy products. With increasing ionic strength, the electrostatic repulsion of stabilized oil droplets decreases, which may cause flocculation and ultimately coalescence [22]. In this set of experiments monovalent and divalent cations, NaCl and $CaCl_2$, were chosen to adjust the emulsions to the similar ionic strength after emulsion preparation. Volume-based mean particle sizes and the zeta-potential of emulsions at different ionic strengths are shown in Fig. 5A and B.

For $CaCl_2$ addition, solely the emulsion with 3 mM $CaCl_2$ was stable and allowed evaluation, as all further concentrations resulted in emulsion breakdown (see Fig. Appendix A3). At this concentration volume-based mean particle sizes of 0.36 ± 0.03 µm (24 h) and 0.33 ± 0.00 µm (7 days) for 0.003 M were measured. Divalent cations like calcium ions can bind to the surfactin molecule and are reported to be responsible for the development of micelles and therefore aggregation of droplets [48,49]. The sensitivity against divalent cations regarding emulsion stability can also be detected with other surfactants like Tween [50]. Moreover, divalent cations can crosslink molecules and induce flocculation and aggregation of single molecules and oil droplets.

In contrast, emulsions treated with NaCl were stable over the range tested and neither coalescence nor phase separation was visually and microscopically observed (see Fig Appendix A3). During storage for 7 days at 4 °C, no significant change ($p < 0.05$) in mean particle size was detected up to 0.15 M NaCl addition. Samples showed volume-based mean particle size values of 0.34 ± 0.00 µm (0.01 M), 0.35 ± 0.02 µm (0.05 M) and 0.32 ± 0.02 µm (0.15 M) after 7 days. Contrary, for emulsions with 0.3 M NaCl the mean particle diameter increased from 0.33 ± 0.01 µm to 0.50 ± 0.00 µm after one week. The highest concentration of 0.5 M NaCl showed a mean particle size of 0.40 ± 0.01 µm after 24 h and 0.48 ± 0.00 µm after 7 days. No major shift in droplet diameter of volume-based particle size distributions was detected with increasing ionic strength (Fig Appendix A4), which was related to a high zeta-potential even at the highest concentration of NaCl (0.5 M) with -33.91 ± 1.28 mV. This repulsive force at high ionic strength is still strong enough to stabilize the emulsion efficiently.

These results are in agreement with observations obtained by Abdel-Mawgoud et al. [28] who reported stable emulsions prepared with surfactin up to 6% NaCl (~ 1 M). However, while other biosurfactants, such as rhamnolipids and sophorolipids, and common surfactants, such as Tween 80 and gum arabic, are also stable in the presence of high NaCl concentrations, other natural emulsifiers are more sensitive to high ionic strengths, such as lecithin (>100 mM NaCl), saponins (>300 mM NaCl), and whey proteins (>200 mM) [9]. In this sense, surfactin showed a comparable resistance towards NaCl than currently used surfactants.

4. Conclusion

The current study demonstrated that purified surfactin from a *B. subtilis* culture was efficient in reducing the interfacial tension at very low surfactant concentration. Interfacial rheological characterization revealed that surfactin emulsions do not form strong viscoelastic interfaces but have a high interfacial charge, which facilitate emulsion stability in the pH-range 6–9 and up to 0.5 M NaCl. Overall, the results demonstrated that surfactin is a strong candidate to form and stabilize food emulsions under the given conditions with comparable or even better performances than currently used natural and synthetic surfactants (*e.g.* lecithins). Further studies are therefore feasible to evaluate the influence of different surfactin congeners and emulsion compositions, as well as sensorial evaluation. In addition, a production using renewable resources or waste-streams should be addressed to pave the way for a sustainable surfactin production.

CrediT authorship contribution statement

Denise Mück performed most of experiments, collected and evaluated corresponding data. **Lena Greiner** and **Peter Klausmann** supported experimental workflow. **Mareen Hoffmann (MH)** and **Lutz Grossmann (LG)** structured and supervised experimental works. MH drafted the whole manuscript. LG substantially contributed to

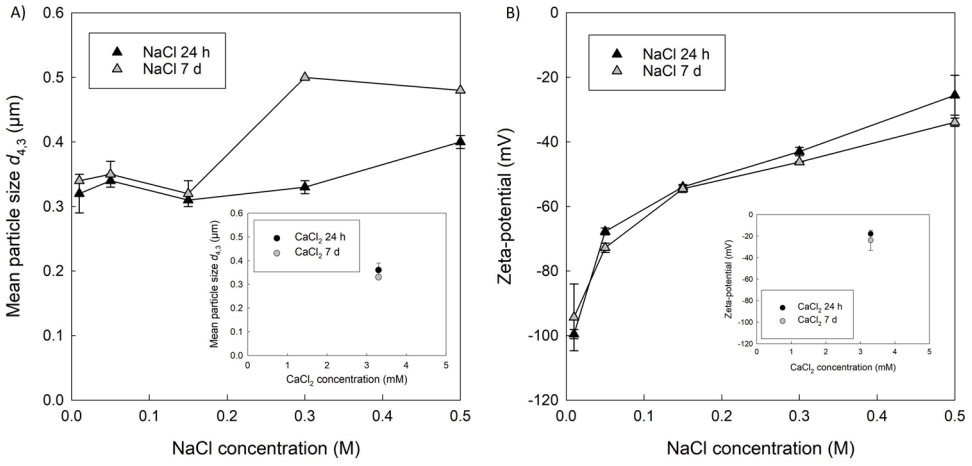

Fig. 5. Influence of ionic strength on the volume-based mean particle size ($d_{4,3}$) (µm) of O/W emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, 500 bar, four cycles) (A), and impact of ionic strength on the zeta-potential (mV) of O/W emulsions (5 g/L surfactin, 10 % (*w/w*) Miglyol oil, pH 7, 500 bar, four cycles) (B).

interpretation of data and results. **Lars Lilge**, **Marius Henkel**, **Jochen Weiss** and **Rudolf Hausmann** read and approved final version of manuscript.

Author contributions

DM performed most of experiments, collected and evaluated corresponding data. LGRE and PK supported experimental workflow. MHO and LGRO structured and supervised experimental works. MHO drafted the whole manuscript. LGRO substantially contributed to interpretation of data and results. LL, MHE, JW and RH read and approved final version of manuscript.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2021.111749.

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