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Microbial Synthesis of Nonionic Long-Chain Sophorolipid Emulsifiers Obtained from Fatty Alcohol and Mixed Lipid Feeding

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Starmerella bombicola **is known to produce sub-terminally hydroxylated lactonic sophorolipids (SLs), while** ***Candida kuoi*** **synthesizes acidic open chain SLs with terminally hydroxylated fatty acids. Upon feeding glucose and fatty alcohols both strains form long-chain nonionic SLs. According to structure elucidation the SLs consist of a hydroxylated fatty acid esterified with fatty alcohol and linked via a glycoside bond to the diacetylated sophorose unit. Palmityl, stearyl, and oleyl alcohols lead to products with lipid chain lengths of C32 or C36. Oleyl alcohol is the preferred substrate leading to 45 g L^{-1} of the double unsaturated C36 SL with** ***S. bombicola*** **and 20 g L^{-1} with** ***C. kuoi*****. Scale up from shake flask to 1.5 L fermentations is possible and 65 g L^{-1} long-chain SLs are obtained with** ***S. bombicola*** **within 7 days. Mixed feeding of oleic acid and a variety of fatty alcohols leads to new long-chain SLs. In the presence of oleic acid the yeasts do not oxidize the fatty alcohol and thus the production of biosurfactants with tailored chain length is possible. The long-chain SLs show good emulsification ability of water/paraffin oil mixtures at low energy input and reduced interfacial tension significantly.**

Practical Applications**: Sophorolipids are produced by fermentation on industrial scale focusing on cleaning and detergent applications. Mainly lactonic or anionic open-chain forms are used today. The new long-chain SLs presented in this manuscript are accessible with existing production technology and can be produced with high titers from cost-efficient renewable raw materials. In contrast to the commercial products the long-chain SLs are more hydrophobic and exhibit a strong emulsification behavior. Therefore they have the potential to broaden the application range of SLs in future. They may be useful as novel emulsifiers for cosmetic creams and lotions, pharmaceutical ointments and food products or may find application in oil spill remediation.**

1. Introduction

Sophorolipids (SLs) are surface-active bio-based glycolipids, which are commercially available for detergent and cleaning applications.[1–3] The non-pathogenic yeast *Starmerella bombicola* is the most studied SL producer well known for its high production titers exceeding 300 g L^{-1}.[4,5] The main SL is a lactonic glycolipid containing a ω-1 hydroxylated fatty acid bound to the diacetylated diglucoside sophorose (**Figure 1**A).[6,7] Structurally divergent acidic open-chain SLs with terminally hydroxylated fatty acids (Figure 1B) were isolated from phylogenetically related yeasts *Candida batistae*[8] and *Candida kuoi*.[9,10] The latter organism exclusively synthesizes the terminally hydroxylated fatty acid and additionally forms some dimeric and trimeric SLs. The preferred substrates for the yeasts are C16 and C18 fatty acids. While these are hydroxylated by a P450 monooxygenase and directly incorporated into the SLs, substrates of other chain lengths are either elongated or metabolized via the β-oxidation pathway.[11–13] Only *Rhodotorula bogoriensis* is known to synthesize a C22 SL, which possesses a branched chain lipid tail hydroxylated at position C13.[14,15]

So far only a few attempts focused on the production of more hydrophobic straight-chain SLs, which may be useful as bio-based emulsifiers. Feeding of C22 methyl esters to *S. bombicola* led to the formation of methylated open-chain SLs, which exhibited lower critical micelle concentrations (CMCs) than the corresponding C18 SLs.[16] The group of Richard Gross used chemical transesterification to obtain long-chain deacetylated SL esters with lipid tails up to C28 from the lactone.[17–19] The CMC values of the esters decreased with increasing chain length[18] and the long-chain esters exhibited good emulsifying properties of several oil/water mixtures.[19–21] Upon enzymatic re-acetylation of the sophorose, diacetylated long-chain esters with a structure closer to that of the native SLs were obtained.[17,22] These esters behaved differently than the corresponding non-acetylated SLs exhibiting increased CMCs at lipid tail lengths of >C22. Stronger head to

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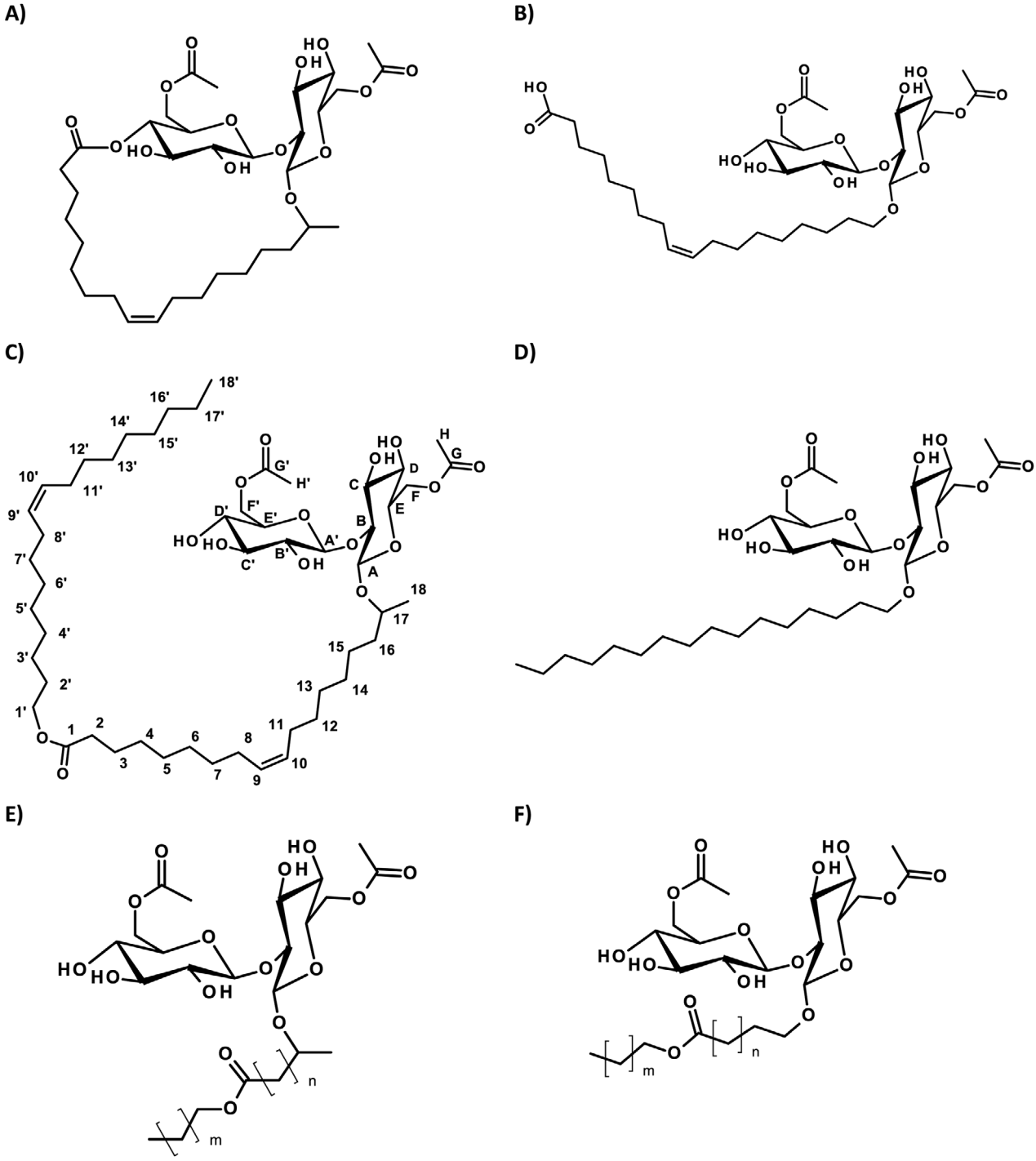

Figure 1. Structures of major SLs from *S. bombicola* A) and *C. kuoi* B) with oleic acid as substrate; C) long-chain SL from oleyl alcohol according to ref. [23] and D) SL with hexadecanol as substrate according to ref. [26]; structures of long-chain esters from mixed oleic acid/alcohol feeding of *S. bombicola* E) and *C. kuoi* F).

head repulsion was the supposed explanation for this unexpected behavior.[22]

The chemical synthesis of diacetylated long-chain SL esters is a multi-step process[17,22] and therefore a direct fermentative route would be advantageous. Early literature from Tulloch and Spencer[23] suggests that feeding oleylalcohol led to the formation of a C36 long-chain ester in *S. bombicola* (Figure 1C). It was assumed that oleyl alcohol was partially oxidized to 17-hydroxy-oleic acid, incorporated into the SL and then esterified with the excess oleyl alcohol. In contrast a direct incorporation of fatty alcohols was reported when either 2-alkanols or 1-dodecanol at a high glucose (150 g L^{-1}) and yeast extract concentration (4 g L^{-1})

were used.[24,25] Similarly direct incorporation of cetyl alcohol was reported leading to a new nonionic SL with anticancer properties (Figure 1D).[26,27] Also β-oxidization and fatty alcohol oxidation deficient strains favored direct incorporation of fatty alcohols into the SLs.[28,29]

The goal of this study was the evaluation of *S. bombicola* and *C. kuoi* for production of long-chain SL emulsifiers according to the structure proposal of Tulloch and Spencer.[23] Especially for *C. kuoi*, which solely incorporates terminally hydroxylated fatty acids into SLs, the direct incorporation of a primary fatty alcohol was assumed an equally probable synthesis route. A variety of fatty alcohols was tested and additionally equimolar fatty acid/fatty alcohol mixtures were examined as substrates. We hypothesized that the P450 monooxygenase[13] catalyzed hydroxylation of the fatty acid is preferred over fatty alcohol oxidation. Therefore production of SLs with tailored lipids by feeding oleic acid in combination with fatty alcohols directly to the microorganisms (Figure 1E,F) should be a feasible alternative to chemical SL modification.

2. Experimental Section

2.1. Materials and Strains

Oleic acid (C18:1, 92.0%, C16:0 2.4%, C18:0 1.0%, C18:2 2.1%) was kindly provided by DAKO AG (Wiesentheid, Germany) and oleyl alcohol (C18:1-OH, 95.5%, C16:0-OH 3.9%), stearyl alcohol (octadecanol, C18:0-OH, 98.7%), and cetyl alcohol (hexadecanol, C16:0-OH, 98.0%, C14:0-OH 1.1%) were provided by BASF Personal Care and Nutrition GmbH (Düsseldorf, Germany). 1-tetradecanol (C14:0-OH, ≥98%), 1-dodecanol (C12:0-OH, ≥98%), 1-decanol (C10:0-OH, ≥99%), and 1-octanol (C8:0-OH, ≥99%) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Solvents (acetonitrile, ethyl acetate, *n*-hexane, DMSO-d6, ≥99%) were purchased from Fisher Scientific GmbH (Schwerte, Germany). *S. bombicola* NRRL Y-17069 and *C. kuoi* NRRL Y-27208 were obtained from the USDA culture collection and were maintained as glycerol stocks at −80 °C. All media components (glucose, yeast extract, malt extract, and peptone) were purchased from AppliChem GmbH (Darmstadt, Germany). HCl-Methanol for GC derivatization was obtained from Sigma Aldrich (United States of America).

2.2. Sophorolipid Production

Biomass growth of *S. bombicola* NRRL Y-17069 and *C. kuoi* NRRL Y-27208 was done in baffled shake flasks filled with growth medium consisting of glucose (20 g L^{-1}), yeast extract (3 g L^{-1}), malt extract (3 g L^{-1}), and peptone (5 g L^{-1}). After 24 h of cultivation at 30 °C the cells were separated from the growth medium by centrifugation at 4 °C, washed twice with water, and resuspended in water. 1 L baffled shake flasks were filled with 0.1 L production medium consisting of 500 mM glucose and 200 mM lipids. The optical density of the cell suspension was measured photometrically and an OD_{600} of 15 was adjusted in each flask. The lipids in the cultures were either oleic acid or fatty alcohols (oleyl alcohol, stearyl alcohol, palmityl alcohol) or an equimolar mixture of oleic acid and primary fatty alcohol (1-decanol, lauryl alcohol (1-dodecanol), myristyl alcohol (1-tetradecanol), palmityl alcohol (1-hexadecanol), or stearyl alcohol (1-octadecanol). Production was run for 14 days at 30 °C under continuous shaking with 150 rpm. After 7 days 250 mM glucose were added to prevent glucose limitation.

Fermentations in 1.5 L scale were done with both strains and palmityl alcohol or oleyl alcohol as substrates. Precultures were grown in 20 and 100 mL shake flasks for 24 h at 140 rpm and 28 °C in an orbital shaker. The biomass was transferred to a BioFlo Celligen 115 fermenter (Eppendorf AG) and grown for 24 h at 28 °C in a medium composed of glucose (20 g L^{-1}), peptone (5 g L^{-1}), yeast extract (3 g L^{-1}), and malt extract (3 g L^{-1}) for biomass production. The cells were then harvested by centrifugation and transferred into nitrogen-limited production medium containing 100 g L^{-1} glucose, 1 g L^{-1} yeast extract, and 60 g L^{-1} oleyl alcohol or 50 g L^{-1} palmityl alcohol respectively. The fermentations were run for 7 days at 28 °C with an aeration rate of 2 vvm, a stirring rate of 1000 rpm and a pH value adjusted to 3.5 with 5 M NaOH. 25 g L^{-1} of glucose were added after 2 and 4 days to prevent glucose limitation.

2.3. Sophorolipid Purification

SLs were extracted from the crude broth with ethyl acetate. One hundred milliliters of ethyl acetate were added to the shake flasks and the solvent was mixed cautiously to prevent extensive emulsification. The phases were separated with a separating funnel and the aqueous phase was extracted again with 100 mL ethyl acetate. The combined ethyl acetate phases were washed with water and then the solvent was evaporated at 40 °C in a rotary evaporator. Residual ethyl acetate and water were removed via lyophilization with an Alpha 2–4 LSC plus freeze drier (Martin Christ GmbH, Germany) at −82.5 °C and 0.05 mbar.

Purification of individual SL species was carried out with an Interchim puriflash 4250/250 preparative HPLC system equipped with a Kromasil 100 C18 column (20 mm × 250 mm, 5 µm) and a PDA detector. SLs were separated with a water—acetonitrile gradient containing 0.1% formic acid. Fractions were analyzed with thin-layer chromatography (TLC) using chloroform/methanol/H_2O in 65:15:2 ratios as mobile phase and silica gel 60 F_{254} as stationary phase. Hanessian's stain (10 g L^{-1} ceric sulfate, 50 g L^{-1} ammonium heptamolybdate tetrahydrate, conc. sulfuric acid 10% v/v, H_2O 90% v/v) was used as detection reagent. Fractions containing identical SL species according to TLC were pooled, lyophilized, and further analyzed via HPLC-ELSD and NMR.

2.4. HPLC-ELSD and HPLC-MS Analysis

SLs were analyzed by HPLC using a Nexera XR system (Shimadzu Corp., Japan) equipped with a LaChrom II C18 reverse phase column (4.6 mm × 250 mm, 5 µm, Hitachi Ltd. Corp., Japan) using a gradient of water (eluent A) and acetonitrile (eluent B) each supplemented with 0.1% (v/v) formic acid at a constant flow rate of 1 mL min^{-1} and 30 °C. The elution conditions

were: initial hold of 50% acetonitrile for 25 min; linear gradient to 100% acetonitrile for 60 min; hold of 100% acetonitrile for 25 min. Detection and quantification of the SLs was done with a ELSD 80 system (VWR Collection, United States) using nitrogen with a pressure of 3.5 bar as nebulization gas at 40 °C. The equipment was controlled by LabSolutions software (version 5.86) from Shimadzu Corp.

Mass spectrometric analyses were performed using a Shimadzu LC-30AD with a SPD M20A diode array detector and a LCMS-2020 single quadrupole mass spectrometer with atmospheric pressure chemical ionization (APCI-MS) equipped with an identical Hitachi LaChrom II C18 reversed phase column (4.6 mm × 250 mm, 5 µm). The mass spectrometer was run in positive and negative scan mode in the m/z range from 150 to 1250 at 10 000 u s^{-1} and an event time of 0.1 s. Corona pin voltage was turned to 4.5 kV. Time program and eluents were the same as in LC-ELSD analysis. Fragmentation patterns were predicted with the CMF-ID prediction tool and compared to the fragmentation patterns observed in positive scan mode (http://cfmid.wishartlab.com/).[30]

2.5. GC Analysis

The hydroxyl fatty acid- and fatty alcohol profiles of the native SL mixtures were examined by gas chromatography according to Konishi et al.[8] 10 mg SL, fatty acids or fatty alcohol were solved in 1 mL 1.25 M HCl in methanol and incubated for 3 h under mixing at 80 °C. Reaction was quenched with 1 mL water and the lipids were extracted with 2 mL *n*-heptane and dried with sodium sulfate. GC analysis of the methyl ester derivatives was done with a Shimadzu GC-2010 Plus system and an MTX-Biodiesel TG column (0.53 mm × 14 m, 0.16 µm) (Restek GmbH, Germany) with a temperature gradient from 75 to 420 °C. Detection was done with a flame ionization detector (FID).

2.6. NMR-Based Structure Elucidation

NMR spectra were recorded with a 400 MHz Bruker Ascend 400 in deuterated dimethyl sulfoxide (DMSO-d6). Besides ^{1}H- und ^{13}C-NMR analysis, 2D-NMR experiments were used for structural analysis. Homonuclear bond correlation was elucidated with COSY experiments, heteronuclear bond correlation with HSQC, and HMBC experiments. The software MestReNova 10.0 (Mestrelab Research S.L.) was used for data analysis.

2.7. Physicochemical Characterization

Native SLs (0.1% w/w) were solved in 75/25 H_2O (pH 9, 0.1% NaCl)/paraffin oil and mixed for 1 min with 80 rpm in a vertical rotating (overhead) shaker. The formation and stability of the emulsions were observed for 60 min. A negative control without any surface active substance was tested as reference system.

Interfacial tensions between water and paraffin oil with native SLs were measured with a tensiometer DCAT 21 from Dataphysics using the Wilhelmy plate method.

3. Results and Discussion

3.1. Sophorolipid Biosynthesis with Glucose and Fatty Alcohols

S. bombicola and *C. kuoi* were cultivated with glucose and palmityl, stearyl, or oleyl alcohol as lipid substrates in comparison to oleic acid as reference substrate. Assignment of individual SL species was done with HPLC-APCI-MS and NMR and SL quantification was done with HPLC-ELSD. Both strains successfully produced SL mixtures with all substrates in titers of 32–78 g L^{-1} (*S. bombicola*) and 4–22 g L^{-1} (*C. kuoi*) (**Table 1**). Despite having the same starting concentration of biomass, product titers with *C. kuoi* were significantly lower in comparison to *S. bombicola*, resulting in residual fatty alcohol at the end of the cultivation. Upscaling of the SL production with palmityl and oleyl alcohol to 1.5 L scale worked well with product titers of 20–84 g L^{-1} (Table 1).

In agreement with literature data both strains produced known SL structures with oleic acid as substrate.[10,31] The main products were isolated by preparative HPLC and identified by NMR as lactonic SL containing a subterminal hydroxylated oleic acid (ω-1) for *S. bombicola* (Figure 1A) and an acidic SL containing terminal hydroxylated oleic acid (ω) for *C. kuoi* (Figure 1B). Shifts of glucose signals at positions C'–E' and oleic acid C1 were used to distinguish lactonic and acidic SL structures (**Table 2**). The ω- and ω-1 hydroxylation pattern was distinguished from signals of positions 16–18. *C. kuoi* additionally produced some dimers and trimers of the acidic SLs (Table 1), which were previously described by *Price* et al.[10]

When using fatty alcohols as substrate, new SL peaks at retention times of >90 min were detected in HPLC-ELSD alongside the known lactonic and acidic SLs (**Figure 2**a). HPLC-APCI-MS mass analysis indicated that the new SL species are composed of diacetylated sophorose with one hydroxylated fatty acid and one fatty alcohol unit. An exemplary mass spectrogram of the SL peak at 97 min from *S. bombicola* fed with oleyl alcohol is shown in Figure 2b. Assuming the proposed structure of Tulloch and Spencer[23] the peak at m/z 958 corresponds to the protonated molecule $[M+H]^+$. The major protonated fragments at m/z 531, m/z 549, and m/z 754 were forecasted by the CMF-ID prediction tool[30] as the most probable structures. These fragments contain a long-chain lipid ester tail as structural motif. For NMR structure elucidation the new SLs were isolated by preparative HPLC. The signals of the sophorose units of the oleylalcohol based SLs were identical to the pattern of the acidic *C. kuoi* SL (Table 2) and no esterification with the sophorose was detected. Thus a diacetylated sophorose linked glycosidically at position D' to the hydroxylated fatty acid can be concluded for both new *S. bombicola* and *C. kuoi* SLs. Specific fatty alcohol derived signals at 64 ppm (C1'), and 31.8, 22.5, and 14.4 ppm (C16'–C18') were only detected in the new SL molecules. Analysis of fatty acid signals at C16–C18 revealed that the hydroxylation pattern was the same as for the oleic acid based SLs. Strict ω-hydroxylation was observed in the case of *C. kuoi* and a preference for ω-1 hydroxylation with *S. bombicola*. In the *S. bombicola* SL some ω-hydroxylated product (C18: 69.1 ppm) was detected as minor component, which could not be separated from the major ω-1 hydroxylated product by prep HPLC purification.

According to mass analyses new long-chain SLs of the same structural composition were produced with all fatty alcohols.

Table 1. Product compositions including mass assignments of the SL mixtures obtained from oleic acid or fatty alcohol transformation after extraction (only SLs were summed up for titer assessment), data from shake flask cultures and fermentations in 1.5 L scale (in brackets), co-eluting minor components in italics, all assigned species were diacetylated.

Substrates [g L^{-1}]	*S. bombicola*				*C. kuoi*			
	Titer [g L^{-1}]	Product composition	Content [%]	m/z $[M+H]^+$	Titer [g L^{-1}]	Product composition	Content [%]	m/z $[M+H]^+$
Glucose (135), Oleic acid (56.4)	78	Acid, C18:1, ω-1	1.4	707	17	Acid, C18:1, ω	65.8	707
		Acid, C18:0, ω-1	0.2	709		Acid, C18:0, ω	2.1	709
		Lactone, C18:1, ω -1	97.2	689		Dimer	17.9	1396
		Lactone, C18:1, ω	0.8	689		Trimer	11.0	2084
		Lactone, C18:0, ω	0.3%	691				
Glucose (135), Palmityl alcohol (48.4) 1.5 L scale: Glucose (150) Palmityl alcohol (50)	32 (33)	Lactone, C16:0, ω-1	1.5 (1.3)	663	4 (20)	Acid, C16:0, ω	1.2	681
		Lactone, C16:0, ω	1.3 (0.3)	663		Hexadecanyl ester, C16:0, ω	93.5 (93.5)	906
		Lactone, C18:1, ω-1	0.4 (0.3)	689		Hexadecanyl ester, C18:1, ω	2.6 (2.5)	906
		Lactone, C18:0, ω-1	0.1(0.1)	691				
		Hexadecanyl ester, C16:0, ω-1	34.3 (46)	906				
		Hexadecanyl ester, C16:0, ω (*and Hexadecanylester, C18:1, ω -1*)	58.4 (49.8)	906 (*932*)				
Glucose (135), Stearyl alcohol (54)	66	Lactone, C18:1, ω-1	5.2	689	17	Acid, C16:0, ω	1.4	681
		Lactone, C18:0, ω-1	84.2	691		Acid, C18:1, ω	12.4	707
		Lactone, C18:0, ω	0.6	691		Acid, C18:0, ω	5.5	709
		Octadecanyl ester, C18:0, ω-1	6.7	962		Octadecanyl ester, C18:1, ω	5.4	962
		Octadecanyl ester, C18:0, ω	0.7	962		Octadecanyl ester, C18:0, ω	68.1	962
Glucose (135), Oleyl alcohol (53,6) 1.5 L scale: Glucose (150), Oleyl alcohol (60)	68 (84)	Acid, C18:1, ω-1	1.2 (1.5)	707	22 (39)	Acid, C18:1, ω	1.2 (30.4)	707
		Acid, C18:1, ω-1	(0.2)	707		Octadec-9-enyl ester, C18:1, ω	91.8 (60.7)	958
		Lactone, C18:1, ω-1	20.2 (13.7)	689				
		Lactone, C18:1, ω	(0.3)	689				
		Lactone, C18:0, ω-1	0.5 (0.5)	691				
		Octadec-9-enyl ester, C18:1, ω-1	52.5 (63.1)	958				
		Octadec-9-enyl ester, C18:1, ω	14.3 (14.4)	958				
		Octadecanyl ester, C18:0, ω-1	1.0 (1.3)	960				

Oxidation of the fatty alcohol gave the hydroxylated fatty acid, which was incorporated into the SL and then esterified with non-oxidized fatty alcohol. Thus the structure proposal of Tulloch and Spencer (Figure 1C)[23] was confirmed by our studies and generally the long-chain SLs had structures according to Figure 1E,F, the majority of them with n = m (Table 1). Probably the lactone esterase of *S. bombicola* is responsible for this esterification reaction as it shares homology with lipase A from *Pseudozyma antarctica*.[32] Presumably a similar esterase is responsible for SL oligomer formation in *C. kuoi* and this enzyme may also catalyze long-chain SL synthesis. A direct incorporation of the fatty alcohols into the SLs, leading to structure D (Figure 1), was not detected in our cultivations. In agreement strict acceptor substrate specificity was reported for *S. bombicola* glucosyltransferases UgtA1.[33]

The content of the hydrophobic long-chain SLs was generally high with a 66–>90% share in shake flask cultures (Table 1) corresponding to, for example, yields of 45 g L^{-1} (*S. bombicola*) and 20 g L^{-1} (*C. kuoi*) of the new oleyl alcohol derived SL species. Only in the case of stearyl alcohol *S. bombicola* produced the

Table 2. NMR structure elucidation data of glucose/oleic acid derived lactonic SL from *S. bombicola* (Sb lactone) and acidic SL from *C. kuoi* (Ck acidic) in comparison to the long-chain SLs obtained from glucose/oleyl alcohol feeding of *S. bombicola* (Sb oleylalcohol) and *C. kuoi* (Ck oleylalcohol), numbering of atoms according to structure C in Figure 1.

Moiety	No.	Sb lactone		Sb oleylalcohol		Ck acidic SL		Ck oleylalcohol	
		$^{\delta}$C [ppm]	$^{\delta}$H [ppm]	$^{\delta}$C [ppm]	$^{\delta}$H [ppm]	$^{\delta}$C [ppm]	$^{\delta}$H [ppm]	$^{\delta}$C [ppm]	$^{\delta}$H [ppm]
Glucose 1 (Glycosyl bond)	A	102.2	4.37	101.5	4.37	101.5	4.32	101.5	4.32
	B	82.8	3.22	83.1	3.26	83.1	3.22	83.1	3.22
	C	76.2	3.42	76.3	3.44	76.2	3.38	76.3	3.38
	D	70.4	3.10	70.2	3.13/3.16	70.0, 70.2	3.11	70.0/70.2	3.12
	E	73.5	3.42	74.3	3.38	74.3	3.32	74.3	3.32
	F	64.0	4.05, 4.23	64.1	4.08/4.27	64.1, 64.2	3.99, 4.23	64.1/64.2	4.01/4.24
Acetate	G	170.7	–	170.6	–	170.7	–	170.7	–
	H	21.1	2.00	21.1	2.04	21.1	2.00	21.1	2.01
Glucose 2 (Ester bond)	A′	104.4	4.50	104.9	4.4	104.7	4.41	104.8	4.4
	B′	75.7	3.10	75.3	3.07	75.2	3.01	75.2	3.01
	C′	73.1	3.42	76.3	3.23	76.3	3.17	76.2	3.17
	D′	70.7	4.71	70.2	3.13/3.16	70.0, 70.2	3.11	70.0/70.2	3.12
	E′	71.7	3.61	73.7	3.42	73.7	3.38	73.7	3.37
	F′	62.7	3.96	64.1	4.08/4.27	64.1, 64.2	3.99, 4.23	64.1/64.2	4.01/4.24
Acetate	G′	170.3	–	170.6	–	170.7	–	170.7	–
	H′	21.0	2.00	21.1	2.04	21.1	2.00	21.1	2.01
Hydroxyoleic acid	1	172.5	–	173.4	–	175.0	–	173.4	–
	2	33.9	2.30	34.0	2.21/2.28	34.1	2.18	34.1	2.18/2.26
	3	24.5	1.52	25.0	1.54	25.0		25.0	1.49
	4-7	28–31	1.1–1.4	28–31	1.1–1.4	28–30	1.1–1.4	28–30	1.1–1.4
	8, 11	26.9, 27.2	2.01, 2.03	27.0	2.00	27.0	2.00	27.0	1.98
	9, 10	129.9, 130.4	5.31	130.0	5.33	130.1	5.33	130.1	5.33
	12–14	28–31	1.1–1.4	28–31	1.1–1.4	28–30	1.1–1.4	28–30	1.1–1.4
	15	25.2	1.24, 1.37	24.9	1.1–1.4	28–30	1.1–1.4	28–30	1.1–1.4
	16	37.5	1.37, 1.46	36.7	1.36/1.54	25.8	1.27	25.8	1.28
	17	78.1	3.61	76.5	3.66 (1.52)	29.7	1.48	29.8	1.48
	18	21.5	1.12	21.7 (69.1)	1.14 (3.48/3.71)	69.1	3.43, 3.66	69.1	3.47, 3.67
Oleylalcohol	1′			64.0	4.01			64.0	3.98
	2′			28.6	1.58			28.6	1,54
	3′			25.9	1.31			25.8	1.27
	4'-7′			28–31	1.1–1.4			28–30	1.1–1.4
	8', 11′			27.0	2.00			27.0	1.98
	9', 10′			130.0	5.33			130.1	5.33
	12'-15′			28–31	1.1–1.4			28–30	1.1–1.4
	16′			31.8	1.26			31.8	1.23
	17′			22.5	1.28			22.6	1.25
	18′			14.4	0.88			14.4	0.85

saturated lactonic SL preferably and only 7.4% of the long-chain SL species were synthesized. Stearyl alcohol is solid at fermentation temperature and insufficient solubilization may be a reason for increased lacton production. Once stearyl alcohol is oxidized and incorporated into SLs, the concentration of solubilized stearyl alcohol may be too low to outcompete lacton formation. Palmityl alcohol is also solid at fermentation temperatures, but on the contrary higher amounts of long chain ester were formed (Table 1). An explanation for this difference may be the substrate acceptance of *S. bombicola*. Ashby et al.[11] found that stearic acid was a significantly better substrate than palmitic acid and more acidic SLs were produced with palmitic acid. Therefore lactonization may be the rate determining step in SL synthesis with palmitic acid and long-chain ester synthesis may be the preferred reaction of the lacton esterase in the presence of palmityl alcohol.

In 1.5 L fermentations, the same SL species were synthesized and from oleyl alcohol 65 g L^{-1} long-chain SL were obtained with *S. bombicola*. The ratio of ω and ω-1 hydroxylation differed to some extent in the *S. bombicola* trials in shake flasks and fermenters. With palmityl alcohol an increased ω hydroxylation was

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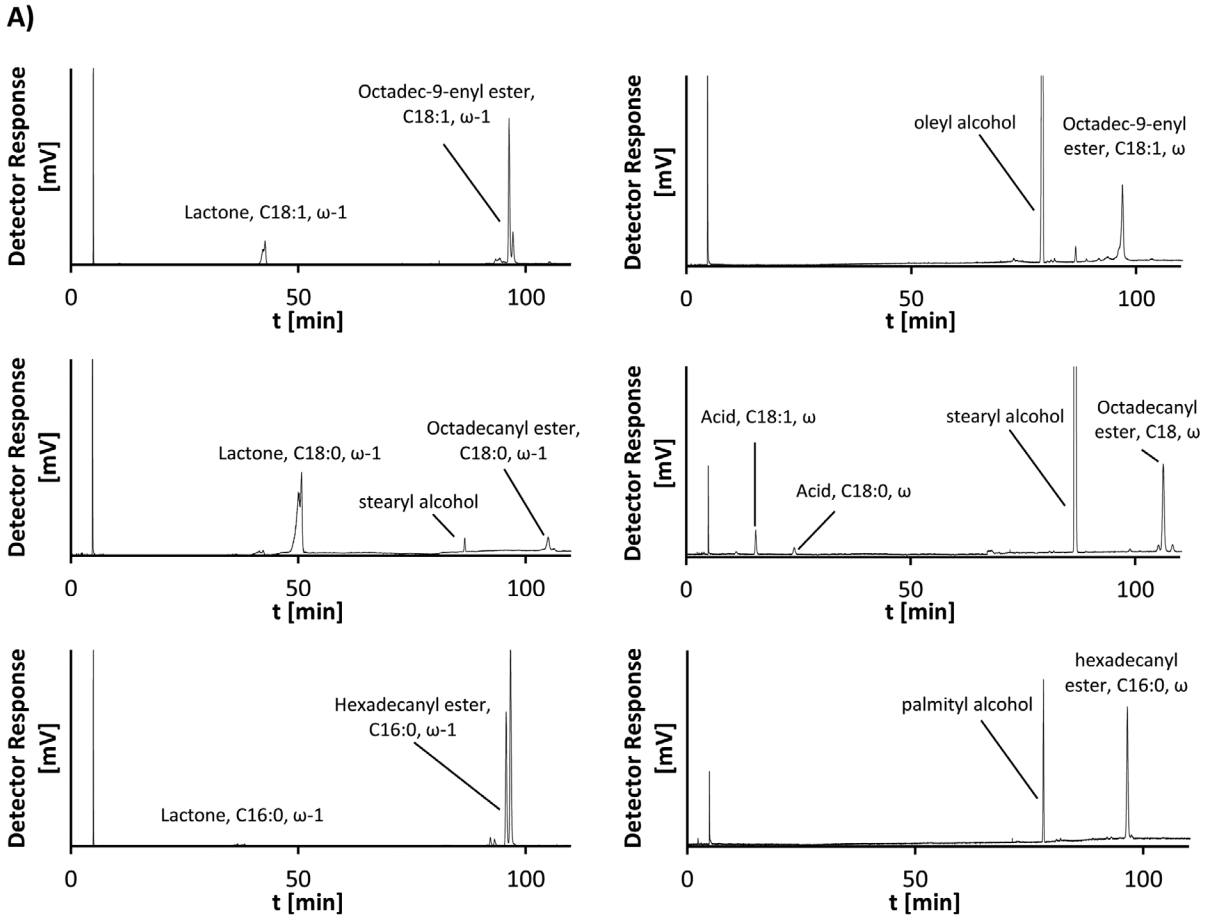


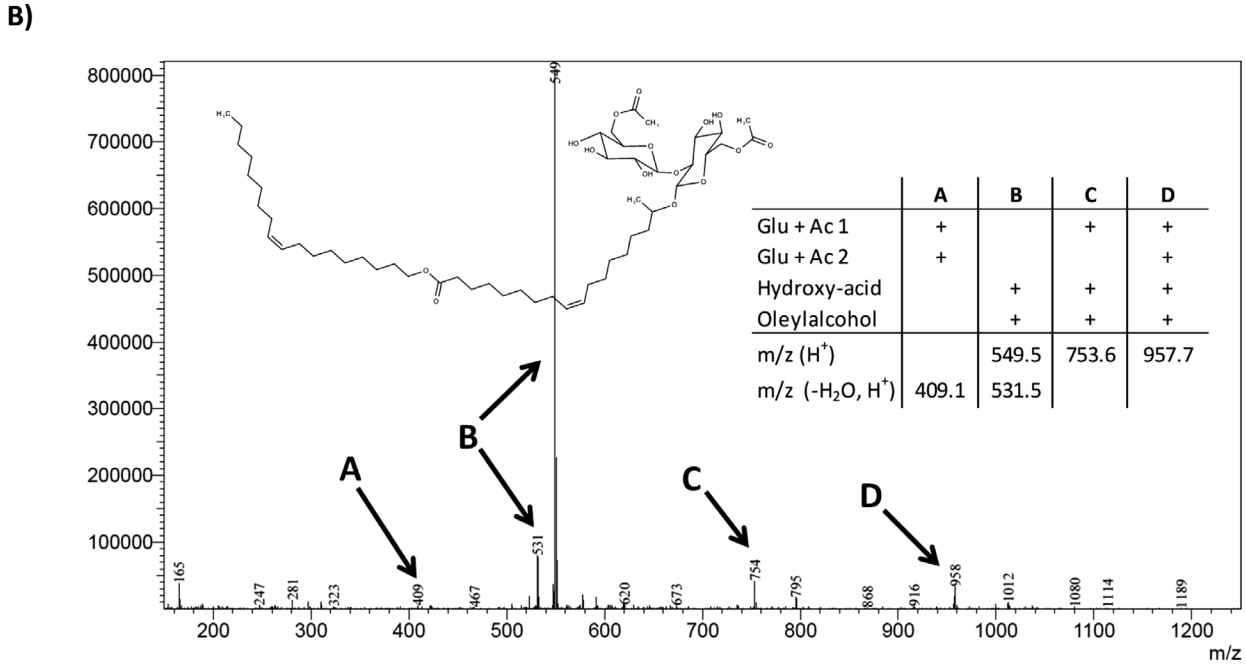
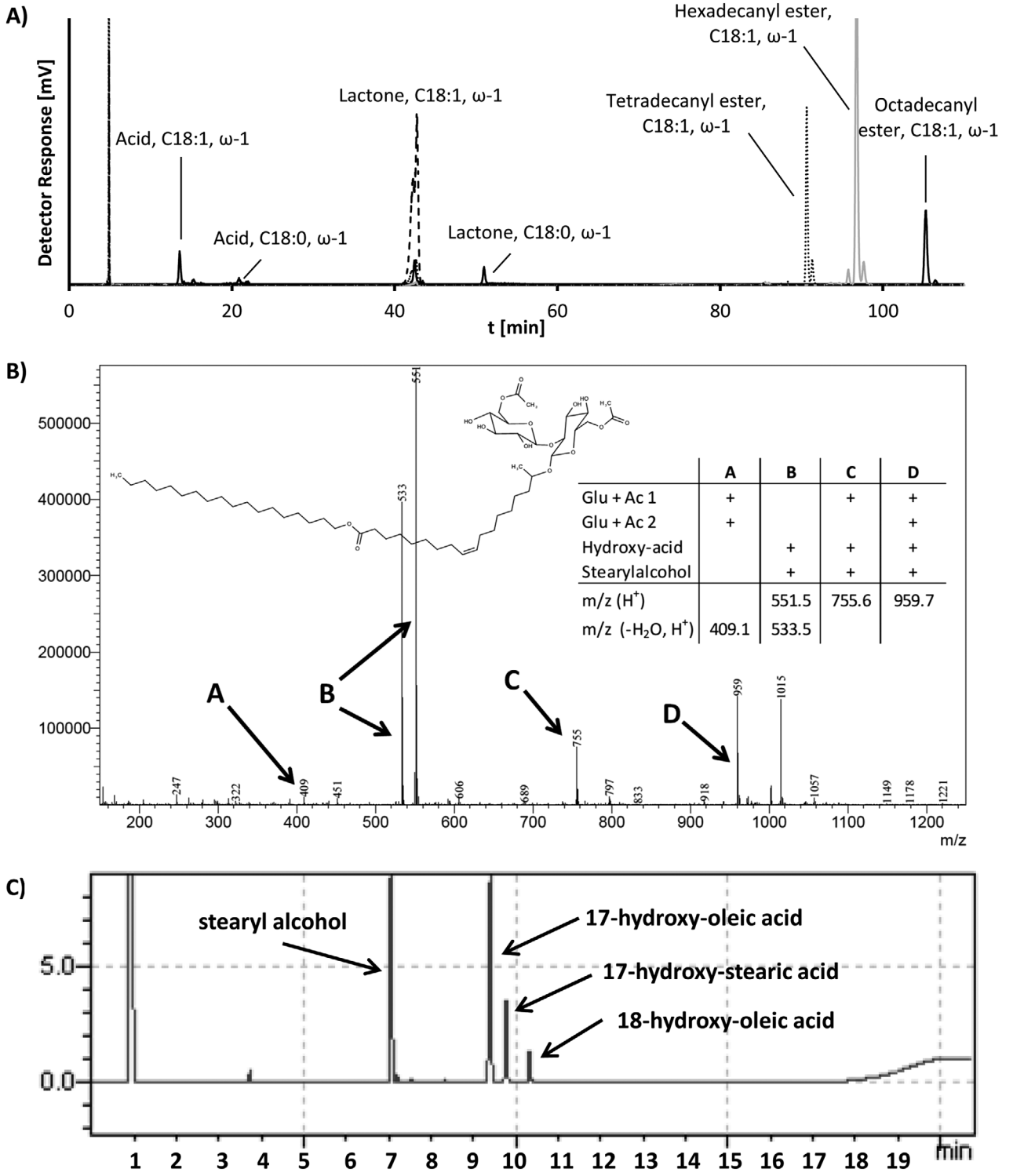


Figure 2. A) HPLC-ELSD overlay of SLs obtained from fatty alcohol feeding of *S. bombicola* (left column) and *C. kuoi* (right column) with oleyl alcohol (top lane), stearyl alcohol (middle lane) or palymityl alcohol (bottom lane); adjacent to the ω-1 hydroxylated products, SLs with ω-hydroxylation are visible in spectra of *S. bombicola* products from oleyl and palmityl alcohol (upper and lower left); B) Positive APCI-MS of the long-chain SL from oleyl alcohol feeding of *S. bombicola* (Octadec-9-enyl ester, C18:1, ω-1) including assignment of the main fragments.



	A	B	C	D
Glu + Ac 1	+		+	+
Glu + Ac 2	+			+
Hydroxy-acid		+	+	+
Stearylalcohol		+	+	+
m/z (H^+)		551.5	755.6	959.7
m/z ($-H_2O$, H^+)	409.1	533.5		

Figure 3. A) HPLC-ELSD overlay of SLs obtained from *S. bombicola* with mixed feeding of oleic acid and fatty alcohols (stearyl alcohol, palmityl alcohol and myristyl alcohol); the peak at 4.5 min represents the dead volume of the HPLC system (elution of salts); small peaks adjacent to the ω-1 hydroxylated products are the compositional equivalent SLs possessing ω-hydroxylation; B) Positive APCI-MS of the long-chain SL from oleic acid and stearyl alcohol feeding of *S. bombicola* (Octadecanyl ester, C18:1, ω-1) including assignment of the main fragments; C) GC analysis of lipids from SL of *S. bombicola* fed with oleic acid and stearyl alcohol obtained from HCl/MeOH splitting (peak assignment was done based on reference GC chromatograms of stearyl alcohol, oleic acid, and lactonic SLs from *S. bombicola*).

Table 3. Product compositions including mass assignments of the SL mixtures obtained from mixed oleic acid and fatty alcohol feeding after SL extraction (only SLs were summed up for titer assessment), data from shake flask cultures, co-eluting minor components in italics, all assigned species were diacetylated.

Substrates [g L^{-1}]	*S. bombicola*				*C. kuoi*			
	Titer [g L^{-1}]	Product composition	Content [%]	m/z $[M+H]^+$	Titer [g L^{-1}]	Product composition	Content [%]	m/z $[M+H]^+$
Glucose (135), Oleic acid (28.2), Stearyl alcohol (27)	61	Lactone, C18:1, ω-1	1.9	689	21	Acid, C18:1, ω	2.5	707
		Lactone, C18:0, ω-1	1.7	691		Octadecanylester, C18:1, ω	63.3	960
		Octadecanylester, C18:1, ω-1 (*and Octadecanylester, C18:0, w-1*)	82.8	960 (962)				
		Octadecanylester, C18:1, ω	9.6	960				
Glucose (135), Oleic acid (28.2), Palmityl alcohol (24.2)	59	Lactone, C18:1, ω-1	1.7	689	6	Acid, C18:1, ω	3.4	707
		Hexadecanylester, C16:0, ω-1	3.6	906		Hexadecanylester, C16:0, ω	60.8	960
		Hexadecanylester, C18:1, ω-1 (*and Hexadecanylester, C16:0, ω*)	84.8	932 (906)		Hexadecanylester, C18:1, ω	34.2	932
		Hexadecanylester, C18:1, ω	6.5	932				
Glucose (135), Oleic acid (28.2), Myristyl alcohol (21.4)	52	Acid, C18:1, ω-1	4.0	707	12	Acid, C18:1, ω	5.3	707
		Acid, C18:0, ω-1	0.3	707		Acid, C18:0, ω	0.7	709
		Lactone, C18:1, ω-1	25.9	689		Tetradecanylester, C18:1, ω	60.5	904
		Lactone, C18:0, ω-1	0.5	691				
		Tetradecanylester, C18:1, ω-1	56.7	904				
		Tetradecanylester, C18:1, ω	09.2	904				
Glucose (135), Oleic acid (28.2), Lauryl alcohol (18.6)	35	Acid, C18:1, ω-1	0.4	707	<1	Acid, C18:1, ω	Traces	707
		Lactone, C18:1, ω-1	97.6	689		Dodecanylester, C18:1, ω	Traces	876
		Lactone, C18:0, ω-1	0.4	691				
		Dodecanylester, C18:1, ω-1	2.9	876				
		Dodecanylester, C18:1, ω	1.1	876				
Glucose (135), Oleic acid 28.2), Decanol (15.8)	19	Acid, C18:1, ω-1	1.3	707	3	Acid, C18:1, ω	0.9	707
		Lactone, C18:1, ω-1	93.1	689		Acid, C18:0, ω	0.6	709
		Lactone, C18:1, ω	0.9	689		Decanylester, C18:1, ω	8.2	848
		Lactone, C18:0, ω-1	0.3	691				
		Decanylester, C18:1, ω-1	0.5	848				
		Decanylester, C18:1, ω	0.1	848				

observed under both conditions. *C. kuoi* produced more acidic SL in 1.5 L fermentations compared to the shake flask trials. Better aeration in the fermenters may increase oxidation rates which could lead to hydroxyl-fatty acid synthesis outcompeting long-chain SL production. Thus, reaction engineering is needed in the large scale for optimized long-chain SL production.

3.2. Sophorolipid Tailoring by Mixed Lipid Feeding

The hydroxylation of fatty acids is catalyzed by the CYP52M1 monooxygenase and it was reported that activity of this enzyme is increased in the presence of glucose and vegetable oil, whereas expression of the monooxgenase responsible for alkane metabolism declines.[34] Similarly fatty alcohol oxidase activity of *S. bombicola* was lower when alkanes were absent.[35] We thus hypothesized that in the presence of fatty acids the alkane oxidization systems of *S. bombicola* and *C. kuoi* are outcompeted by CYP52M1 and fatty acid hydroxylation is the preferred reaction in a mixture of oleic acid and primary fatty alcohol. Concordantly upon feeding equimolar concentrations of oleic acid and fatty alcohols of varying chain lengths the production of several new SLs was observed (**Figure 3**A). With decreasing fatty alcohol chain lengths, shorter retention

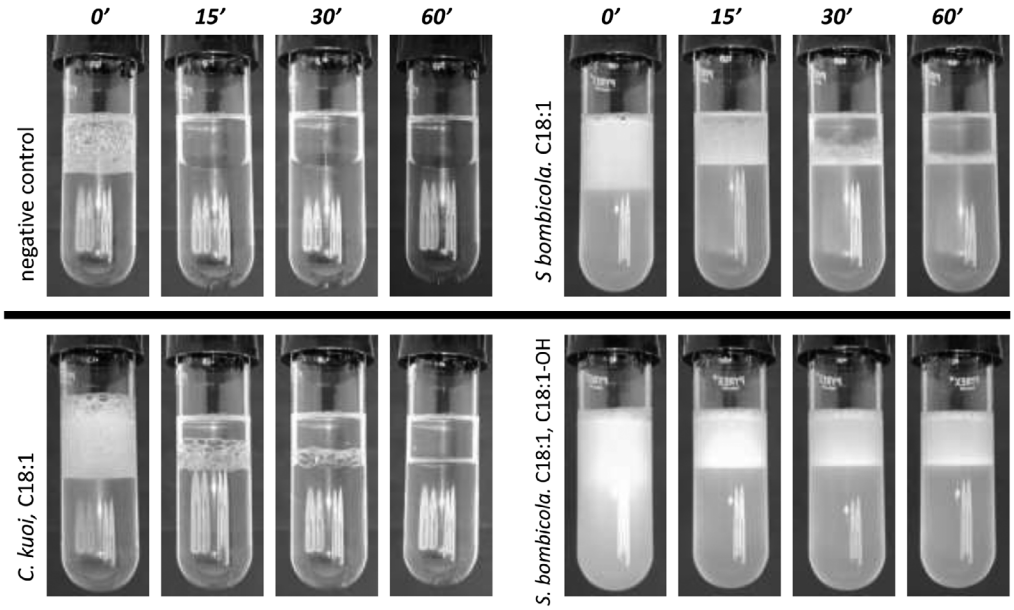


Figure 4. Emulsification stability trials with a 75/25 H_2O/paraffin oil mixture; negative control (without surface active substance) (top left), lactonic SL (top right), acidic SL (bottom left), long-chain SL (bottom right), the SL mixtures as outlined in Table 1 were used.

times in HPLC were observed corresponding to a decreased hydrophobicity.

Analysis of mass fragmentation of the new SL obtained from oleic acid and stearyl alcohol feeding revealed major fragments with a mass difference of 2 in comparison to the fragmentation pattern of the oleyl alcohol derived SL (Figure 3B). The major protonated fragments were forecasted by the CMF-ID prediction tool and pointed to the formation of a long-chain ester. NMR structure elucidation of the SLs derived from *S. bombicola* feeding with oleic acid and stearyl alcohol or palmityl alcohol gave the same signals as the oleyl alcohol derived SL and verified the incorporation of the fatty alcohol. Precise quantification of double bond intensities (two double bonds from oleyl alcohol SL versus one double bond from mixed feeding SL) was not possible, however. Broad overlapping signals from the hydroxyl protons between 5 and 5.6 ppm with double bond protons at 5.3 ppm and overlapping of methyl protons in the region of 2 ppm with protons adjacent to the double bond (positions 8, 11) made quantification impractical. Further analysis of the lipid composition of the new SL was therefore done by GC after acidic transesterification. Major products were stearyl alcohol and hydroxylated oleic acids with preference for the ω-1 site for the *S. bombicola* product based on oleic acid/stearyl alcohol feeding (Figure 3C). Additionally, 17-hydroxy stearic acid was detected, which originates from stearyl alcohol oxidation. 18:1/18:0 and 18:0/18:0 long chain SL species co-eluted in HPLC (**Table 3**) and were obtained in a ration of approximately 80:20 according to GC peak areas (Figure 3C). Mass analysis of the other mixtures revealed that oleic acid was preferably hydroxylated and tailoring of the lipid tail was possible by variation of the fatty alcohol (Table 3) According to HPLC-ELSD and HPLC-MS analysis (Figure 3A) SLs of structure 1F (Figure 1) were produced by *C. kuoi* and SLs of structure 1E were preferably synthesized by *S. bombicola*.

Fatty alcohols with chain lengths from C10 to C18 were fed to the yeast strains. SLs were produced with both strains and all substrates ranging in titers from 19 to 61 g L^{-1} (*S. bombicola*) and <1 to 31 g L^{-1} (*C. kuoi*) (Table 3). As with fatty alcohol feeding alone *C. kuoi* produced SLs less efficiently and non-complete consumption resulted in comparably low titers and residual substrates in the SL phase. The proportion of long-chain SLs was high, when C14–C18 fatty alcohols were used and 56 g L^{-1} of stearyl alcohol based and 54 g L^{-1} of palmityl alcohol based mixed long-chain SLs were obtained with *S. bombicola*. In contrast to stearyl alcohol feeding the mixed lipid feeding led to a significantly higher concentration of long-chain SLs. Oleic acid most probably serves as solubilizer for stearyl alcohol making it better available for incorporation into SLs. A drop in SL production and share of long-chain SLs was observed with fatty alcohols of C10 and C12 chain lengths. These substrates are potentially toxic for the yeasts and clearance via β-oxidation[28] may be an explanation for the low yields. Substrate feeding strategies to reduce toxic effects may improve SL production with alcohols of chain lengths below C14.

3.3. Initial Analysis of Emulsion Stability and Interfacial Tension

While most publications reported the emulsification behavior of SLs with high energy input (shear homogenizer),[19–21] we already observed an excellent emulsification ability during the purification steps of the long-chain SL esters. Therefore the stabilization of a paraffin oil/water mixture with oleyl alcohol based SL ester from *S. bombicola* was tested in a system with low energy input. Lactonic SL from *S. bombicola* and acidic SL from *C. kuoi* were used for comparison. **Figure 4** shows the physical appearance of 0.1 wt% SL emulsions immediately after formation and after 15,

30, and 60 min. The more hydrophobic character of lactonic SL from *S. bombicola* and the long-chain SL lead to little surfactant precipitation and therefore a slightly turbid water phase. In contrast to the acidic and the lactonic SL the long-chain SL did not show bulk separation of paraffin oil after 60 min. The long-chain SL-based emulsion was even stable for at least 5 days. The opacity and cream height also remained unchanged during the test series. Thus long-chain SL ester is most suitable for the emulsification of water/paraffin oil even at low mechanic forces.

Lactonic SL from *S. bombicola* and acidic SL from *C. kuoi* lowered the interfacial tension (IFT) in a water/paraffin oil system down to values of 3.6 ± 0.1 mN m^{-1} with the lactone and 10.8 ± 0.2 mN m^{-1} with the acidic form at concentrations of 50 mg L^{-1} (details will be reported elsewhere). With the long-chain SLs obtained from oleyl alcohol (18:1/18:1) and from oleic acid + palmityl alcohol feeding (18:1/16:0) the IFT dropped to below 1 mN m^{-1} at concentrations of 50 mg L^{-1} in the oil phase. These initial data show the good interfacial activity of the long-chain SLs, well suited for emulsifier applications. Detailed analysis of the interfacial behavior with other methods is needed to determine the exact values.

4. Conclusions

Feeding of fatty alcohols or equimolar fatty acid/fatty alcohol mixtures led to the biosynthesis of novel long-chain SLs. The products obtained from *S. bombicola* and *C. kuoi* differed in their ω- or ω-1-hydroxylation and were accessible in good yields of up to 65 g L^{-1} from 1.5 L fermentations and over 40 g L^{-1} from shake flask experiments. Mixed oleic acid / fatty alcohol feeding enabled tailoring of the lipid moiety. The strategy represents an alternative to chemical modification and is especially suited for very long-chain SL production. The novel biosurfactants exhibited good emulsification properties and may broaden the sophorolipid application range.

Abbreviations

C. kuoi, *Candida kuoi*; *S. bombicola*, *Starmerella bombicola*; SL, Sophorolipid

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biosurfactants, *Candida kuoi*, emulsifiers, fatty alcohols, sophorolipids, *Starmerella bombicola*

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