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From bumblebee to bioeconomy: Recent developments and perspectives for sophorolipid biosynthesis

Sven Dierickx, Martijn Castelein, Jelle Remmery, Veerle de Clercq, Sofie Lodens, Niki Baccile, Sofie L de Maeseneire, Sophie L.K.W. Roelants, Wim K Soetaert

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5 **Author names and affiliations**

6 Sven Dierickx ¹, Martijn Castelein ¹, Jelle Remmery ¹, Veerle De Clercq ¹, Sofie Lodens ¹, Niki
7 Baccile ², Sofie L. De Maeseneire ¹, Sophie L. K. W. Roelants ^{1,3,a}, Wim K. Soetaert ^{1,3}

8 ¹ Centre for Industrial Biotechnology and Biocatalysis (InBio.be), Department of Biotechnology,
9 Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

10 ² Centre National de la Recherche Scientifique, Laboratoire de Chimie de la Matière Condensée de
11 Paris, Sorbonne Université, Place Jussieu 4, 75005 Paris, France

12 ³ Bio Base Europe Pilot Plant, Rodenhuisenkaai 1, 9042 Ghent, Belgium

13 ^a corresponding author

14 E-mail (in order of authorship): sven.dierickx@ugent.be, martijn.castelein@ugent.be,
15 jelle.remmery@ugent.be, veedcler.declercq@ugent.be, sophie.lodens@ugent.be,
16 niki.baccile@sorbonne-universite.fr, sophie.demaeseneire@ugent.be, sophie.roelants@ugent.be,
17 wim.soetaert@ugent.be

18 **Abstract**

19 Sophorolipids are biobased compounds produced by the genera *Starmerella* and *Pseudohyphozyma*
20 that gain exponential interest from academic and industrial stakeholders due to their mild and
21 environmental friendly characteristics. Currently, industrially relevant sophorolipid productivities are
22 reached up to 3.7 g·L⁻¹·h⁻¹ and sophorolipids are used in the personal care and cleaning industry at
23 small scale. Moreover, applications in crop protection, food, bioflotation and medical fields are being
24 extensively researched. The research and development of sophorolipids is at a crucial stage. Therefore,
25 this work presents an overview of the state-of-the-art on sophorolipid research and their applications,
26 while providing a critical assessment of scientific techniques and standardisation in reporting. In this
27 review, the genuine sophorolipid producing organisms and the natural role of sophorolipids are
28 discussed. Subsequently, an evaluation is made of innovations in production processes and the
29 relevance of *in-situ* product recovery for process performance is discussed. Furthermore, a critical
30 assessment of application research and its future perspectives are portrayed with a focus on the self-

31 assembly of sophorolipid molecules. Following, genetic engineering strategies that affect the
32 sophorolipid physiochemical properties are summarised. Finally, the impact of sophorolipids on the
33 bioeconomy are uncovered, along with relevant future perspectives.

34 **Keywords**

35 Sophorolipid, biosurfactants, *Starmerella*, process engineering, genetic engineering, natural role,
36 application research, self-assembly, bioeconomy

37 **1 Introduction**

38 In solving worldwide problems caused by human activity, like global warming or the looming loss of
39 biodiversity (Cardinale et al., 2012; Masson-Delmotte et al., 2019), help can come from unexpected
40 places. Indeed, the smallest organisms will help to make big impact, as micro-organisms produce
41 biochemicals with applications in a plethora of domains like human health, agriculture, waste
42 processing and even transport; and these production processes are much more eco-friendly compared
43 to their chemical counterparts and start from renewable resources (Khoo et al., 2016). Particularly
44 interesting biochemicals are the secondary metabolites, as these compounds have an extensive
45 structural variety resulting in a broad spectrum of properties and activities and therefore can substitute
46 or extend the current markets. Academic and industrial interest in a specific type of secondary
47 metabolites is increasing exponentially, namely sophorolipids (SLs). Sophorolipids consist of a
48 hydrophilic sophorose moiety linked by a glycosidic binding to a fatty acid tail (figure 1). The
49 sophorose head can have up to two acetylation groups while the tail commonly consists of 16 to 20
50 carbon atoms and is saturated to polyunsaturated. SLs can be lactonised due to a condensation reaction
51 between the fatty acid carboxyl group and the sophorose 4'' hydroxyl group (figure 1A). A second
52 sophorose moiety can be linked to the fatty acid tail via an ester binding. The resulting molecules are
53 then referred to as bolaform sophorolipids (figure 1B). It should be noted that SLs are predominantly
54 produced in varying mixtures of different congeners, depending on culture conditions and producing
55 organisms (see section 2). As SLs are amphiphilic molecules consisting of a hydrophilic 'head' and
56 aliphatic 'tail', SL application studies and their commercialisation currently focus on their role as a
57 biosurfactant.

58 *Figure 1: Chemical structures of sophorolipid congeners. R_1 and $R_2 = H$ or OAc A) Lactonic SL*
59 *($C_{18:1}$, $\omega-1$) B) Bolaform SL ($C_{18:1}$, $\omega-1$) C) Acidic SL ($C_{18:1}$, $\omega-1$) D) "branched" SL ($C_{22:0}$, $\omega-10$)*

60 The increasing (industrial) interest in SLs can be illustrated by the exponential increase in
61 patents on SLs published from 2010 till now (figure 2A). To gain more insight in the interested
62 industrial fields, an analysis of the top 10 patent classes according to the international patent
63 classification (IPC) over time was performed. Per time period, the top 10 IPC classes were grouped in

64 six fields, four according to the applications they claim (biocide, medical, cosmetic or detergent
65 applications) and two regarding production methods (organic chemistry or industrial biotechnology)
66 (figure 2B). Regarding production, the top 10 IPC classes are located mainly in the field of
67 fermentation processes, while for the applications cosmetics and detergents predominate. In the period
68 of 2011-2015, IPC classes concerning biocide applications were positioned in the top 10 but they
69 dropped out in 2016-2019. As one patent can belong to multiple IPC classes and only the top 10 is
70 analysed, it should be noted that figure 2 only shows a trend of the most frequently occurring IPC
71 classes. Moreover, this analysis does not take in to account the impact on the sophorolipid
72 development per single patent. Hence, important fields can be neglected (e.g. genetic engineering).

73 *Figure 2: Average annual counts were collected in the Lens patent database with the search term*
74 *“sophorolipid*” A) Average annual count of published patents per time period B) Average annual*
75 *count of top 10 IPC patent codes per time period. IPC codes classified per parent class: biocide*
76 *application (A01N25/30, A01N43/16, A01P3/00), medical application (A61K31/70, A61K31/7016,*
77 *A61K31/7024, A61K31/739), cosmetic application (A61K8/00, A61K8/60, A61K8/73, A61Q19/00,*
78 *A61Q19/10, A61Q5/02), organic chemistry (C07H15/04, C07H15/06, C07H15/10), detergent*
79 *application (C11D1/66, C11D11/00, C11D3/00, C11D3/20, C11D3/22, C11D3/30, C11D3/37,*
80 *C11D3/386, C11D3/42), fermentation process (C12P19/12, C12P19/44, C12P7/62) (Cambia, 2020)*

81 Sophorolipids are industrially relevant molecules and are clearly the subject of a large number of
82 recent research papers and patent applications. However, an expanding field can suffer from success as
83 incorrect methodologies and the lack of a complete overview can propagate to persistent academic and
84 industrial misunderstandings and failures. Furthermore, the link between academic research and
85 industrial developments is often ignored or incorrect information on industrial developments and
86 requirements is presented. Therefore, this review provides the reader a complete overview and critical
87 assessment of research and industrial development. Moreover, important aspects that are neglected in
88 previous sophorolipid concerning reviews are tackled in this work. More specifically, the self-
89 assembly of sophorolipid molecules which is the main driver for its application potential and the
90 natural role of sophorolipids.

91 This work is structured to guide the expanding sophorolipid community towards efficient
92 research and commercialisation. To aid researchers, this review provides a critical assessment of
93 scientific techniques used in the field of SLs and proposes a standardisation for reporting. Both
94 academic and industrial stakeholders will benefit from the state-of-the art overviews on producing
95 micro-organisms and their respective SL production spectrum (section 1), unravelled biological
96 functions (section 2), advances in SL production process development (section 3), SL applications
97 (section 4), genetic engineering strategies (section 5) and insights on the industrial potential of SLs
98 and their impact on the bioeconomy (section 6).

99 2 Producing micro-organisms

100 Although otherwise reported in the past, all sophorolipid producing organisms are members of the
101 *Starmerella* clade (genera *Starmerella*), except *Pseudohyphozyma bogoriensis*. Though closely
102 related, except *P. bogoriensis*, these yeasts differ in SL production spectrum.

103 2.1 Acknowledged sophorolipid producers

104 The very first observation of SL production was reported by Gorin et al. (Gorin et al., 1961) in
105 1961 with the ascomycetous yeast *Starmerella apicola*, which was originally isolated from sow thistle
106 petals. This yeast was formerly known as *Torulopsis magnoliae*, *T. apicola* and *Candida apicola*
107 (Santos et al., 2018; Tulloch and Spencer, 1968) and produces a heterogeneous SL mixture which
108 mainly consists of lactonic SLs of which the mono- and non-acetylated forms are abundant, and which
109 contains minor amounts of the acidic congeners (Kurtzman et al., 2010). The (C_{18:1}) hydroxy fatty acid
110 group is mainly ω-1 linked to the sophorose head group (Price et al., 2012).

111 In 1968, Tulloch et al. first described the production of a specific type of acidic sophorolipids
112 by *Candida bogoriensis*, namely 13-[(2'-0-13-D-glucopyranosyl-8-n-glucopyranosyl)-oxy]
113 docosanoic acid 6',6"-diacetate (figure 1D) (Tulloch et al., 1968). Later on, this yeasts' name was
114 altered to *Rhodotorula bogoriensis* (Nuñez et al., 2004), and recently it was changed again, to
115 *Pseudohyphozyma bogoriensis* (Wang et al., 2015). *P. bogoriensis* was first isolated from the leaf
116 surface of the *Randia malleifera* shrub in Indonesia. The SLs produced by *P. bogoriensis* contain 13-
117 hydroxydocosanoic acid (13-OH-C₂₂) as the lipid moiety (Solaiman et al., 2015) and are also called
118 'branched C₂₂ SLs'. Also, minor amounts of C₂₄ branched SLs are present in the mixture (Ribeiro et
119 al., 2012a). Both the 'branched' structure and the length of the incorporated fatty acids is unique
120 among SL-producing organisms.

121 *Starmerella bombicola* (initially referred to as *Torulopsis bombicola* or *Candida bombicola*)
122 was first isolated from the nectar of a bumblebee in 1970 (Spencer et al., 1970). It became the best
123 known and the most intensively studied SL producing yeast species, due to its naturally high
124 production titres (> 200 g·L⁻¹) (Davila et al., 1997; Gao et al., 2013; Van Bogaert et al., 2015; Zhang et
125 al., 2018) and high overall productivities (up to 3.7 g·L⁻¹·h⁻¹) (Gao et al., 2013). Indeed, *S. bombicola*
126 is the only SL producing yeast of which the (entire) SL production pathway has been elucidated
127 (figure 5 and described in section 6) (Ciesielska et al., 2014; Saerens et al., 2010; K. M. Saerens et al.,
128 2011b; K. M. J. Saerens et al., 2011c; Van Bogaert et al., 2013). Typically, SLs produced by *S.*
129 *bombicola*, but also by other strains of the *Starmerella* clade, contain a hydrophobic hydroxy fatty acid
130 moiety that is ω-1 hydroxystearate (C_{18:0}), ω/ω-1 hydroxyoleate (C_{18:1}) or hydroxylinoleate (C_{18:2}), but
131 ω/ω-1 intermediates with 16 C-atoms (C_{16:0} and C_{16:1}) have also been detected (Ashby et al., 2008;
132 Tulloch et al., 1962). These hydroxy fatty acid moieties are linked to a sophorose moiety. The SLs
133 may be acetylated at the 6' and/or 6" positions of the sophorose moiety (Tulloch et al., 1967) and a

134 macrocyclic lactone structure may form between the 4" hydroxyl group of the sophorose molecule and
135 the carboxyl group of the hydroxy fatty acid moiety (Tulloch et al., 1967). In contrast to other species
136 within the *Starmerella* clade, *S. bombicola* predominantly produces a di-acetylated C_{18:1} lactone form
137 in addition to a minor fraction of the free acid form, but this ratio is susceptible to variation based on
138 culture conditions (see below). Although the SLs of *S. bombicola* mainly consist of a C_{18:1} hydroxy
139 fatty acid, (sub)terminally (ω -1) linked to a sophorose molecule, a clear structural diversity in the
140 produced SL mixtures has been demonstrated. Kurtzman et al. (2010) proved this to be the case for the
141 whole *Starmerella* clade.

142 In 1987, another SL producing yeast strain was reported, namely *Starmerella floricola*
143 (formerly known as *Candida floricola*), which was isolated from dandelion and azalea flowers
144 (Tokuoka et al., 1987). In 2010, Imura et al. (2010) described its ability to produce SLs. These SLs are
145 predominantly di-acetylated C_{18:1} acidic SLs. This was confirmed in recent papers by Konishi et al.
146 (2017, 2018), as new *S. floricola* isolates were reported to primarily produce ω -1 C_{18:1} di-acetylated
147 acidic SLs at a concentration of approximately 36.1 g·L⁻¹ and with a productivity of 0.21 g·L⁻¹·h⁻¹ in
148 shake flask experiments when glucose and oleic acid were fed simultaneously.

149 *Starmerella batistae* (formerly known as *Candida batistae*) was first described in 1999. It was
150 isolated from larval provisions, larvae, and pupae of the solitary bees *Diadasina distincta* and *Ptilotrix*
151 *plumata* (*Apidae*) in Minas Gerais, Brazil (Rosa et al., 1999). The SLs produced by *S. batistae* consist
152 of 75% ω -hydroxy fatty acids (mostly C_{18:1} and to a very small extent some C_{18:0} and C_{18:2} congeners),
153 which is different from the SLs produced by *S. bombicola*, consisting of 65% - 72% (ω -1)-hydroxy
154 fatty acids (Konishi et al., 2008; Inge N A Van Bogaert et al., 2009). Also, *S. batistae* typically
155 produces more than 60% acidic SLs, in contrast to *S. bombicola*, which produces more than 65%
156 lactonic SLs. It can thus be concluded that *S. batistae* primarily produces C_{18:1} terminally linked (ω)
157 diacetylated acidic SLs (Konishi et al., 2008).

158 In 2010, Kurtzman et al. (2010) evaluated several strains of the *Starmerella* clade for SL
159 production, including *S. stellata*, *S. riodocensis* and *Candida* sp. Y-27208, later identified as
160 *Starmerella kuoi* (Kurtzman, 2012). *S. stellata* is a common isolate from grape must and can be used
161 in a co-fermentation with *S. cerevisiae* for wine production (Soden et al., 2000). *S. riodocensis* was
162 isolated from pollen-nectar provisions, larvae and faecal pellets of *Megachile* sp. bees (Pimentel et al.,
163 2005), whereas *S. kuoi* was isolated from concentrated grape juice in Cape Province, South Africa
164 (Kurtzman, 2012). These three strains produce very little lactonised SLs compared to *S. bombicola*
165 and *S. apicola*. The major SL produced by these three species is the di-acetylated acidic C_{18:1}
166 congener, along with smaller amounts of mono- and non-acetylated acidic C_{18:1} SLs (Kurtzman et al.,
167 2010; Kurtzman et al., 2011; Price et al., 2012). *S. kuoi* and *S. riodocensis* produce ω linked SLs,

168 while for *S. stellata* it was not specified whether the hydroxy fatty acid was ω or ω -1 linked to the
169 sophorose molecule.

170 2.2 *Controversial sophorolipid producers*

171 Besides the eight SL producing species mentioned above, which are thoroughly identified and
172 for which the structure of the SLs they produce is thoroughly studied, also some other, more doubtful
173 reports of SL producers were reported, which are critically discussed below.

174 In 2006, Chen et al. first reported SL-production by the yeast *Wickerhamiella domercqiae*,
175 which was isolated from oil containing wastewater (not further defined) (Chen et al., 2006).
176 Subsequently, the same group investigated the influence of several nitrogen (N) sources on growth and
177 SL production and composition (Ma et al., 2012, 2011). The formation of acidic SLs was stimulated
178 when inorganic N-sources such as ammonium sulphate were used, whereas the use of organic N-
179 sources promoted the formation of lactonic SLs. Later, they also investigated the influence of metal
180 ions on SL production: addition of Mg^{2+} ions promoted the production of lactonic SLs, whereas Fe^{2+}
181 ions promoted the production of acidic SLs (Chen et al., 2014). A patent application for this selective
182 production of either acidic either lactonic SLs was filed in 2014 (Chen and Zhang, 2014; Song, 2013).
183 Several other papers were published about the effects of different factors on the SL production with
184 this species (Li et al., 2013, 2012; Liu et al., 2016). However, in the beginning of 2016, strain *W.*
185 *domercqiae* Y2A CGMCC 3798, reported in the articles and patent, was reclassified as *S. bombicola*
186 based on sequence analysis, meaning the conclusions apply to *S. bombicola* instead of *W. domercqiae*
187 (Li et al., 2016). This clearly underlines the importance of thorough strain characterisation.

188 SL production was reported in 2008 for *Wickerhamomyces anomalus*, formerly known as
189 *Pichia anomala* PY1 (Punrata et al., 2020; Thaniyavarn et al., 2008). LC-MS analysis of the product
190 revealed molar mass peaks that could be correlated to $C_{20:0}$ and $C_{18:1}$ SLs. Yet, Souza et al. (2017)
191 concluded from NMR analysis that the molecules produced by this strain cannot contain a sophorose
192 moiety. However, a full identification was not accomplished. Nevertheless, *W. anomalus* might not
193 produce SLs, since the molecules claimed to be SLs might not contain a sophorose moiety, as
194 described by Souza et al. (2017).

195 *Candida rugosa* and *Rhodotorula mucilaginosa*, isolated from hydrocarbon contaminated
196 sites, were shown to produce biosurfactants in the presence of 2% (v/v) diesel as sole carbon- and
197 energy source (Chandran and Das, 2011). It was claimed that *C. rugosa* produces mono-acetylated
198 lactonic $C_{18:1}$ SLs and that *R. mucilaginosa* produces di-acetylated acidic $C_{18:1}$ SLs. It was not
199 specified whether the hydroxy fatty acid chains are coupled via the ω or the ω -1 position. However,
200 the proposed structures should be reconsidered as the reported mass to charge ratios of m/z 728 and
201 m/z 668 do not correspond to those of the $C_{18:1}$ SLs. Though the reported molar masses might be
202 consistent with sodium adducts of $C_{18:1}$ congeners (Ribeiro et al., 2012b), this should be checked

203 before conclusions can be drawn. In addition, the identification of these two yeasts is doubtful, as it
204 was done using a Vitek yeast card reader, which is not particularly accurate. Chandran and Das (2012)
205 described SL production by *Candida tropicalis* in 2012. The yeast was isolated from contaminated soil
206 in India and reported to produce SLs in the presence of diesel oil, making it an interesting and efficient
207 diesel oil degrader. However, the same remarks as to their study on *C. rugosa* and *R. mucilaginosa* can
208 be made. Strain identification is doubtful and though the reported mass to charge ratio m/z of 668
209 could correspond to the sodium adduct of lactonic $C_{18:1}$ SL (Ribeiro et al., 2012b), they claim it
210 corresponds to $C_{20:4}$ mono-acetylated lactonic SLs, which is doubtful. Moreover, the reported titre and
211 productivity only amount to $1 \text{ g}\cdot\text{L}^{-1}$ and $0.003 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, respectively, rendering this strain not
212 particularly interesting for SL production at industrial scale.

213 The production of SLs by *Cyberlindnera samutprakarnensis* was first reported in 2013
214 (Poomtien et al., 2013). The strain was isolated from cosmetic industrial waste in Thailand. In the
215 presence of 2% glucose and 2% palm oil it produced $1.89 \text{ g}\cdot\text{L}^{-1}$ crude SLs with a mean volumetric
216 productivity of $0.0113 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ at shake flask level. The authors claimed production of non-acetylated
217 lactonic ω -1 $C_{18:0}$ SL and di-acetylated lactonic ω -1 $C_{16:0}$ SLs linked to mass over charge ratios of 574
218 and 662 in a MALDI-TOF-MS analysis. However, a mass over charge ratio of 574 does not
219 correspond to non-acetylated lactonic $C_{18:0}$ SLs (m/z 606.3604 [M-H]), so what is actually produced
220 should be further investigated. It could be suggested that they are $C_{16:2}$ non-acetylated lactonic SLs.
221 Furthermore, the chromatogram shows a mass of 664 instead of 662. The m/z of 664 might correspond
222 to $C_{18:1}$ mono-acetylated acidic SLs instead of the claimed di-acetylated lactonic $C_{16:0}$ SLs.

223 *Candida albicans* 0-13-1 was described for the first time as a SL-producing yeast in 2012
224 (Yang et al., 2012). A high titre of $108 \text{ g}\cdot\text{L}^{-1}$ was reported. In the reported mixture mostly $C_{18:1}$ di-
225 acetylated lactonic SLs were detected, although also the $C_{18:2}$ and $C_{18:0}$ variants were found. Later, the
226 same research group published a new bioreactor design, enabling integrated SL production using *C.*
227 *albicans* 0-13-1 (Zhang et al., 2018). However, as there are no other reports claiming SL production
228 by *C. albicans*, it is possible that these authors are actually dealing with another strain as it was in
229 neither of the two publications mentioned how the producing strain was identified.

230 In 2014, Basak et al. found that *Cryptococcus* sp. VITGBN2 is a potent producer of SLs in
231 mineral salt media containing vegetable oil as additional carbon source to glucose. The chemical
232 structure of the purified biosurfactant was proposed to be di-acetylated acidic $C_{18:1}$ SL. It was not
233 mentioned whether the $C_{18:1}$ chain is ω or ω -1 linked to the sophorose head. This study lacks thorough
234 structure identification analysis by e.g. NMR or MS fragmentation. For example, the MW of the
235 extracted biosurfactant is ambiguous as a m/z value of 706 is reported while the unionised
236 monoisotopic mass of a di-acetylated acidic $C_{18:1}$ SL is 706.3776 Da. Though the information of the
237 ionisation mode is lacking, it is highly unlikely that this biosurfactant is a di-acetylated acidic $C_{18:1}$ SL.

238 Sen et. al. (2017) reported on SL production in a novel yeast strain, *Rhodotorula babjevae*
239 YS3, which was isolated from an agricultural field in Assam, Northeast India. A SL titre and a mean
240 volumetric productivity of $19.0 \text{ g}\cdot\text{L}^{-1}$ and $0.26 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ were observed, respectively. The production
241 performance might be further increased by optimisation of the process parameters. The product was
242 characterised as a heterogeneous SL product containing lactonic and acidic congeners after analysis
243 through TLC, FTIR and LC-MS. The produced mixture was claimed to contain non-acetylated acidic
244 $\text{C}_{11:0}$ and $\text{C}_{13:1}$ SLs, as well as non-acetylated lactonic $\text{C}_{13:1}$, $\text{C}_{15:3}$, $\text{C}_{16:0}$, $\text{C}_{18:2}$ SL and di-acetylated
245 lactonic $\text{C}_{18:0}$ SLs (Sen et al., 2017). However, these conclusions should be treated very critically, as a
246 recent publication of another group claims production of extracellular polyol esters of fatty acids
247 (PEFA) by this microorganism *R. babjevae*. (Garay et al., 2017), which they also concluded earlier
248 (Cajka et al., 2016). In the latter publication, Garay et al. claim that the masses of the polar functions
249 of the studied molecules point towards sugar alcohols (polyols) instead of hexoses, which were
250 observed in the SL control they used. It is thus doubtful that this species is a SL-producer.

251 Recently, a new SL-producing microorganism *Lachancea thermotolerans* BBMCZ7FA20 was
252 isolated from the gut of honeybee, *Apis mellifera* (Mousavi et al., 2015). The authors report the
253 production of both acidic and lactonic SLs, but their conclusions are based solely on FTIR analysis.
254 Production titres went up to $24 \text{ g}\cdot\text{L}^{-1}$ in a shake flask experiment when adding $100 \text{ g}\cdot\text{L}^{-1}$ glucose and
255 $100 \text{ g}\cdot\text{L}^{-1}$ canola oil as substrates; however, more detailed analyses are vital in order to unequivocally
256 confirm the identity of these SLs.

257 Specifications on the products produced by all above mentioned organisms are given in table
258 1. It is clear that several alleged SL producing organisms were doubtfully identified, or the structural
259 analysis of the SLs was not sufficiently in depth, thus, most likely not all claimed SL producers are
260 actual SL producers. This illustrates that both proper strain identification and proper SL structure
261 identification is of uttermost importance (Claus and Van Bogaert, 2017). “In the perspective of
262 stringent strain identification, we recommend the use of PCR amplification of reference sequences
263 followed by DNA sequencing and subsequent use of Basic Local Alignment Search Tools (BLAST) to
264 nucleotide sequence databases. Hitherto, all described sophorolipid producers are yeast species and
265 therefore, we recommend the analysis of the internal transcribed spacer (ITS) region in the 18S
266 subunit of ribosomal RNA as reference sequence for species identification. However, when strain
267 identification is aimed, we recommend whole genome sequencing and subsequent sequence alignment
268 (Twigg et al., 2020). Our recommendations for standardisation of sophorolipid structure identification
269 are described in section 4.”

270 *Table 1: Overview of fully acknowledged SL producers and produced SL variants. DiAc = di-*
271 *acetylated, MAc = mono-acetylated, NAc = non-acetylated, ID = identification; Purple hexagon =*
272 *glucose, red filled circle = COOH, red open circle = COO, green line = glycosidic bound (cfr. figure*

273 5). Left schematic represents C18:1 acidic sophorolipids, right schematic represents C18:1 lactonic
274 sophorolipids. a: C18:0, Sls with C16:0 and C16:1 backbones, b: small amounts of Sls with C18:0
275 and C18:2, c: Mac and DiAc C22:0 hydroxylated at C13. Black square = acknowledged, X = not
276 acknowledged, ± = more information is necessary.

277 3 Natural role(s)

278 Many yeasts of the *Starmerella* clade are associated with (bumble)bees or with substrates that are
279 often visited by (bumble)bees. It is thus believed that there is a mutually beneficial relationship
280 between (bumble)bees and various yeast species from the *Starmerella* clade (Rosa et al., 2003).
281 Although many papers and patents deal with production and application of sophorolipids and their
282 producing microorganisms, the natural role of these secondary metabolites for their natural producers
283 remains elusive. Why do these yeasts, found in a very specific niche, produce these non-essential
284 secondary metabolites?

285 The fact that Sls are secondary metabolites indicates they do not fulfil (an) essential
286 function(s) in cell growth and/or -maintenance, but that their presence favours the producing organism
287 in specific conditions, resulting in an evolutionary benefit which sustains production (Hommel and
288 Ratledge, 1993). The elucidation of the natural role(s) of Sls thus leads back to the question about the
289 evolutionary benefit of these molecules for the producing organism. The benefits associated with the
290 production of other biosurfactants are as diverse as the comprising chemical structures and the
291 microorganisms producing them: e.g. the cyclic lipopeptide surfactin shows antimicrobial properties
292 and is believed to be involved in the sporulation of *B. subtilis*; rhamnolipids are linked to the virulence
293 of *P. aeruginosa* by playing a role in biofilm formation and cell motility; and flocculosin, produced by
294 the fungus *P. flocculosa*, was postulated to serve as a storage compound besides being an antifungal
295 agent (Abdel-Mawgoud et al., 2010; Grossman, 1995; Kitamoto et al., 2002; Mimee et al., 2009; Ron
296 and Rosenberg, 2001; Van Hamme et al., 2006).

297 For Sls in particular, the hypotheses that have been postulated since their discovery 60 years
298 ago can be summarised in five theories, but unambiguously supporting evidence is sparse (Roberto De
299 Oliveira et al., 2014; Van Bogaert et al., 2007): (1) Sls improve the uptake of hydrophobic substrates;
300 (2) SL production constitutes an overflow metabolism; (3) Sls exert antimicrobial activity, thereby
301 inhibiting the growth of competing microorganisms; (4) SL production is a protection mechanism
302 against high osmotic pressure in their natural environment; and (5) Sls serve as an extracellular
303 storage compound for carbon and energy.

304 All but one SL producing organisms are members of the *Starmerella* clade: *S. apicola*,
305 *S. bombylicola*, *S. floricola*, *S. batistae*, *S. stellata*, *S. riodocensis* and *S. kuoi* (Konishi et al., 2016;
306 Kurtzman et al., 2010). In nature, these ascomycetous yeasts are found, as some names suggest, in

307 close relation with (bumble)bees and flowers, environments rich in sugars (*e.g.* honey and nectar) and
308 lipids (*e.g.* beeswax and plant oils) (De Graeve et al., 2018; Rosa et al., 2003). Most research papers
309 that deal with the elucidation of the natural role of SLs are focussing on *S. bombicola*, as it is the most
310 investigated SL producing organism. However, it can be assumed that the SLs produced by the other
311 species of the *Starmerella* clade have similar natural functions, due to a shared ecological niche. The
312 exception to the rule is the basidiomycete *P. bogoriensis* – not only aberrant in its produced SL
313 structures, but also in phylogeny and habitat. Although only being isolated twice (from the leaf
314 surfaces of an unidentified *Schefflera* species and *Randia malleifera* in the same botanical garden in
315 Bogor, Indonesia), it is very likely that the natural habitat of *P. bogoriensis*, presumably the
316 phylloplane (the leaf surface of a plant, an oligotrophic environment characterised by the presence of
317 lipids in the cuticle), is different from that of the *Starmerella* species. This is confirmed by the fact that
318 basidiomycetes present in nectar samples were unable to reproduce and were believed to be
319 occasionally present there due to carry-over from the phylloplane (Brysch-Herzberg, 2004; Deinema,
320 1961; Lindow and Brandl, 2003; Ruinen, 1963). Accordingly, the SLs produced by *P. bogoriensis*
321 might have a (slightly) different natural function.

322 3.1 Uptake of hydrophobic substrates

323 The oldest hypothesis on the natural role of SLs dates back to 1982 (Ito and Inoue, 1982). It
324 presumed the involvement of SLs in the uptake of water-insoluble alkanes, because the addition of *e.g.*
325 octadecane to the *S. bombicola* culture medium ‘induced’ SL production. For hexadecane improved
326 growth was observed when a wildtype SL mixture (termed ‘safflower-SL’) was added to the culture
327 medium, seemingly by shortening the lag phase with several days (roughly estimated from about 8 to
328 2 days). Later on, it turned out that *S. bombicola* not only produces SLs in the presence of
329 hydrophobic substrates, but also when grown solely on glucose as C-source (Hommel et al., 1994).
330 Hence, if the natural function of SLs is enhancing the uptake of hydrophobic substrates, the yeast
331 would waste energy in unrequired SL production in the absence of alkanes. On top, the previously
332 reported shortened lag phase for growth on hexadecane could not be repeated by De Clercq et al.
333 (2021); on the contrary: growth on hexadecane declined for both the wildtype and the $\Delta cyp52M1$
334 strain (non SL producing *S. bombicola* strain) when SLs were supplemented to medium with
335 hexadecane as sole carbon source (De Clercq et al., 2021). On the other hand, the addition of SLs to
336 $\Delta cyp52M1$ cultures promoted growth (significantly increased growth rates compared to $\Delta cyp52M1$
337 cultures without SLs addition) on triglycerides (rapeseed oil), which thus indicates that SLs could
338 confer their producing microorganism with a competitive benefit as TGAs are hydrophobic substrates
339 that are present in the natural habitat of *S. bombicola* (De Clercq et al., 2021).

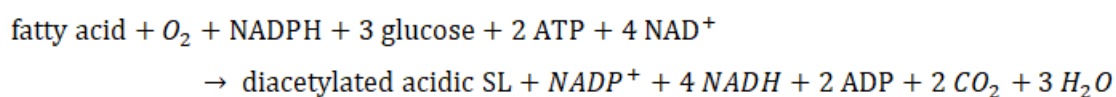
340 Aside from improving growth, the presence of SLs could also enhance the uptake of
341 hydrophobic substrates like triglycerides, which can be incorporated as fatty acids in newly produced
342 SLs and hence reinforcing its own SL production as combined uptake of hydrophilic and hydrophobic

343 substrates results in more efficient SL production. With SLs having additional benefits as described
344 below thus resulting in a competitive advantage. Although many researchers have shown that SLs
345 have emulsifying/solubilising properties, which can enhance the contact of the cells with water-
346 insoluble hydrophobic substrates, the fact that *S. bombicola* produces SLs in the absence of
347 hydrophobic substrates, mainly in the stationary phase and in rather high concentrations, indicates that
348 improved uptake of hydrophobic substrates for growth may not be the main natural role of SLs, which
349 might be different for *P. bogoriensis*. However, the progeny of SL producing cells (which typically
350 arise in the neighbourhood of the original SL producing cells) could benefit from the SLs produced
351 and secreted in the extracellular space by their mother cells i.e. allowing them to grow faster and
352 produce SLs more efficiently on hydrophobic substrates compared to the progeny of non-SL
353 producing cells. This could thus give rise to an evolutionary benefit for SL producing cells over non-
354 SL producing cells.

355 3.2 *Overflow metabolism*

356 A second posed natural role is that SL production constitutes an overflow metabolism in order
357 to regulate the intracellular energy level and redox balances. Davila *et al.* (1997) proposed this theory
358 to clarify the observation that SLs are excreted upon nitrogen starvation. However, overflow
359 metabolism relates to a deficit in the cofactor NAD⁺, caused by excessive NADH formation during
360 glycolysis in fast growing cells, and results to incomplete oxidation of substrates (*e.g.* glucose), even
361 in the presence of oxygen (Szenk *et al.*, 2017). To restore the NAD cofactor balance, overflow
362 metabolites such as lactate, acetate and ethanol are excreted. Several reasons can be alleged why SL
363 production does not fit with this hypothesis. First of all, SLs are typically produced in the stationary
364 phase where the growth rate declines and secondly, the overall biosynthesis reaction of SLs generates
365 NADH instead of recycling it back to NAD⁺ (see biosynthetic reaction 1, which is concurrent with the
366 calculations of Linton as 1 NADPH corresponds to 4 ATP (Linton, 1991)). When generalising the
367 trigger of overflow metabolism to overall redox balances, the oxidation of NADPH to NADP⁺ during
368 (sub)terminal hydroxylation of the fatty acid (the first step in the SL pathway, see figure 5) could help
369 to restore a deficit in NADP⁺. However, NADP⁺ can easily be recovered via reactions in the central
370 metabolism with formation of mannitol (NADP⁺ recycling) - together with ethanol and glycerol
371 (NAD⁺ recycling) - the true end product(s) of the overflow metabolism in *S. bombicola* (Gonçalves *et al.*,
372 2018).

373 *Biosynthetic reaction 1: overall reaction of the formation of di-acetylated acidic SLs (C_{18:1}) starting*
374 *from glucose and fatty acid (C_{18:1}):*



375

376 3.3 *Antimicrobial activity*

377 In a third hypothesis, the natural role of SLs is linked to their antimicrobial activity, inhibiting
378 the growth of microorganisms occupying the same ecological niche as the producing organisms.
379 Antimicrobial activity was first reported for lactonic SLs of *S. bombicola*, inhibiting growth of some
380 alkane utilising yeasts such as *Y. lipolytica* and *C. albicans* (Ito et al., 1980). Other studies
381 substantiated this inhibiting effect not only for yeasts, but also for bacteria (e.g. *B. subtilis* and *P.*
382 *aeruginosa*) and fungi (e.g. *U. maydis*) (De Clercq et al., 2021; Díaz De Rienzo et al., 2016; Gross and
383 Shah, 2003; Haque et al., 2016; Kim et al., 2002; Lydon et al., 2017; Tran, 2012). More recently, also
384 antifungal activity of C₂₂ SLs of *P. bogoriensis* was observed for the first time, in a plate assay with *P.*
385 *acnes* (Solaiman et al., 2015). Important to note is that these antimicrobial experiments were
386 performed with eye on application potential of SLs and therefore, the examined strains lack relevance
387 with the ecological niche of SL producing strains. Nevertheless, SLs indeed exert antimicrobial
388 activity by changing or rupturing cellular membranes through similar effects exerted by detergents and
389 hence target a fundamental and universal prerequisite for survival of cells (Akemi et al., 2018). In this
390 way, the microorganisms populating the natural habitat are likely also inhibited by the presence of
391 SLs, giving the SL producing microorganism a competitive advantage and thus resulting in a niche
392 protection role of SLs.

393 3.4 *Protection against osmotic pressure*

394 A theory far less examined, stated by Hommel *et al.* (1994), proposes that SLs are produced as
395 an adaptation to osmotic stress, arising from the prevalence of sugars in honey and nectar, present in
396 the natural habitat of SL producing microorganisms (with exception of *P. bogoriensis*). Hommel *et al.*
397 noted parallels in the biosynthesis of the sophorose moiety of SLs in *S. apicola* and trehalose synthesis
398 in *S. cerevisiae* (Hommel et al., 1994). The latter is known to act as a compatible solute under
399 (osmotic) stress conditions (Babazadeh et al., 2017). Yet, compatible solutes are defined as highly
400 water soluble and low molecular weight molecules that accumulate to high intracellular
401 concentrations; three conditions that are not fulfilled for SLs (Sleator and Hill, 2002). Inverting this
402 rationale on the other hand, could theoretically imply a relief of osmotic stress: the osmotically active,
403 small sugars outside the cell are converted into higher molecular and less soluble SLs, thereby
404 alleviating the osmotic pressure on the microbial cell. If this process represents the natural function of
405 SLs, it should entail enhanced growth in high sugar concentrations of a strain capable of making SLs
406 compared to a SL deficient strain (e.g. $\Delta cyp52MI$ (Van Bogaert et al., 2013) or $\DeltaugtA1$ (K. M. J.
407 Saerens et al., 2011c)). However, no significant differences in growth (neither in lag, μ_{max} or
408 maximal cell concentration) of the wild type SL producing strain compared to the mutant $\Delta cyp52MI$
409 non-SL producing *S. bombicola* strain were observed when cultured (under laboratory conditions) on
410 glucose or fructose concentrations up to 600 g·L⁻¹ (De Clercq et al., 2021).

411 3.5 *Exclusive storage compound*

412 A fifth and last hypothesis is that SLs serve as an extracellular storage compound of carbon
413 and energy. This was also first stated by Hommel *et al.*, (1994) as trehalose not only functions as a
414 compatible solute but can also act as a storage compound for *S. cerevisiae* (Hommel *et al.*, 1994; Jules
415 *et al.*, 2008). This implies that *S. bombicola* can use its own SLs as carbon source, as was also already
416 suggested by Garcia-Ochoa *et al.* (1996), who filed a patent claiming SLs were degraded from 5 to 1
417 g.L⁻¹ when used as sole carbon source, followed by the formation of sophorose (Garcia-Ochoa and
418 Casas, 1996). Although later on some other reports stated that *S. bombicola* cannot dissimilate SLs
419 (Hu, 2000; Lo and Ju, 2009), Li *et al.* (2016) recently identified a monooxygenase enzyme MoA that
420 could be involved in the metabolism of acidic SLs. They found specificity of MoA towards C_{18:2} di-
421 acetylated acidic SLs, as overexpression or deletion of *moA* only affected the peak area of that
422 compound, with a decrease or increase, respectively. Unfortunately, they used UV absorbance as a
423 detection method, thereby missing the effect of MoA on SLs comprised of saturated fatty acids. The
424 activity and specificity of MoA was confirmed by a heterologous enzyme test with purified MoA
425 enzyme and C_{18:2} di-acetylated acidic SLs as substrate (no activity was found towards C_{18:1} di-
426 acetylated acidic SLs or C_{18:2} di-acetylated lactonic SLs). The authors believe MoA is involved in the
427 catabolism of SLs as they claim the detection of (acetylated) sophorose and hydroxylated fatty acids in
428 the final reaction mixture of the MoA enzyme with C_{18:2} di-acetylated acidic SLs. The latter results
429 need thorough reinvestigation as the data show some inconsistencies (varying mass differences for
430 loss of water, no standards included in the analyses, no negative control as reference, ...). Still, to
431 invigorate the hypothesis that *S. bombicola* is able to catabolise its own produced SLs, our research
432 group found that SLs disappear from the medium during prolonged incubation upon starvation. It was
433 shown that the predominantly produced di-acetylated lactonic SLs are 1. deacetylated and 2. that ring
434 opening occurred, resulting in the extracellular accumulation of non-acetylated acidic SLs. Further
435 experimental evidence indicates subsequent uptake of non-acetylated acidic SLs by the yeast cells and
436 further intracellular degradation thereof into glucose and hydroxylated fatty acids. It was also proven
437 that these reactions are not due to 'spontaneous' hydrolysis, but that certain yet unidentified
438 extracellular and intracellular enzymes of *S. bombicola* are responsible for the observed conversions.
439 This mechanism allowed yeast cells to remain viable in submerged 'starved' conditions for over 3
440 months. Furthermore, our lab confirmed that SLs can also sustain growth when used as sole carbon
441 source (De Clercq *et al.*, 2021).

442 The theory of SLs as extracellular storage compound can also hold for *P. bogoriensis*, as the
443 disappearance of its branched SLs from old culture medium was already reported in 1961 (Deinema,
444 1961). In agreement with our findings, gradual disappearance of di-acetylated SLs occurred after 3 or
445 6 days of cultivation of *P. bogoriensis* and simultaneous appearance of mono- and non-acetylated
446 derivatives was observed (Esders and Light, 1972; Ribeiro *et al.*, 2012a). An acetyltransferase capable of
447 performing these deacetylation reactions was identified some years later by Bucholtz *et al.* (Bucholtz

448 and Light, 1976). They presumed a preference for initial deacetylation of the outer 6'' C of sophorose,
449 which was also noted in more recent research, but further elucidation of the degradation mechanism of
450 SLs in *P. bogoriensis* is still lacking (Bucholtz and Light, 1976; Solaiman et al., 2015).

451 3.6 *Sophorolipids: an exclusive storage compound with antimicrobial properties*

452 To summarise, the most coherent and proven theory on the main natural function of SLs is that
453 they encompass a dual function resulting in an evolutionary benefit to competing microorganisms, *i.e.*
454 as extracellular storage compound with inherent antimicrobial activity. SLs do not protect the
455 microorganism in unfavourable conditions (*cfr.* overflow metabolism and osmotic pressure) and are
456 not primarily produced to enhance growth on hydrophobic substrates, but SLs aid the producing
457 organism to compete against other microorganisms in the ecological niche (*cfr.* antimicrobial activity
458 and storage compound). Valuable and easily degradable carbon sources, such as sugars and/or fatty
459 acids, are claimed by converting them into more inert compounds: SLs. By that, microorganisms
460 create a 'personal hoard' that can be addressed under starvation conditions while minimising nutrient
461 competition with other microorganisms. For *Starmerella* species, these starvation conditions could
462 arise during the hibernation of bumblebee queens, as was speculated by De Clercq *et al.* (2021). By
463 catabolising the previously produced and exclusive SLs, the yeast can keep up its cell numbers,
464 entailing a benefit towards other bumblebee associated microorganisms. Additionally, the
465 antimicrobial activity of SLs could pertain to a possible mutualistic relation between (bumble)bees and
466 SL producing yeasts by inhibiting pathogens.

467 *Figure 3. Overview of the confirmed (in green), refuted (in red) and precarious (in orange) theories*
468 *on the physiological role of sophorolipids for S. bombicola. In nature, this yeast can be found in close*
469 *association with flowers and (bumble)bees. The prevailing theories aroused from environmental*
470 *factors characterising/present in this habitat: high sugar concentrations, a wide variety of*
471 *hydrophobic substrates and the presence of other microorganisms (De Clercq et al., 2021).*

472 4 **Advances in production process optimisation**

473 Apart from being produced by *Starmerella* in its natural environment, SLs are also produced in
474 bioreactors to obtain them as industrially interesting compounds. There is a large abundance of
475 research describing methods towards increasing sophorolipid titres and productivities in submerged
476 bioprocesses. Prior to reviewing the published research regarding this topic, we would like to stress
477 that it is very difficult to compare research outcomes because multiple parameters typically differ
478 between manuscripts and in the authors long-lasting experience, small differences in parameters can
479 make a dramatic difference in production outcomes (productivity/titre related, but also on the
480 distribution of the different congeners). Moreover, the lack of standardisation of the declared
481 production parameters and erroneous quantification and qualification results of the produced SLs often
482 gives rise to more questions than answers.

483 Specifically, as a first issue we would like to address terminology, typical parameters to
484 measure and compare production process outcomes are amounts (g), endpoint titre ($\text{g}\cdot\text{L}^{-1}$), substrate
485 yield ($\text{g}_{\text{SL}}\cdot\text{g}_{\text{substrate}}^{-1}$), productivity ($\text{g}\cdot\text{h}^{-1}$) and volumetric productivity ($\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$). Some research articles
486 solely report an endpoint titre. Yet, mean volumetric productivities and substrate yields can easily be
487 calculated and hence should be declared in addition to the endpoint titre. A high production titre
488 obtained in a very lengthy process for example will be a lot less industrially relevant compared to a
489 lower titre obtained in a short process time. Furthermore, 'titre' or 'concentration' is often referred to
490 as 'yield', which should be avoided. Standardisation in the terminology will allow more
491 straightforward comparison between production processes.

492 A second issue is the use of erroneous quantification techniques, as also critically reviewed
493 recently by Twigg et al. (2020). Crude techniques like gravimetric or colorimetric methods are often
494 used (Hans-J. Daniel et al., 1998; Hans-Joachim Daniel et al., 1998; Pekin et al., 2005), but result in an
495 overestimation of produced SLs. The so-called gravimetric methods consist of heating the
496 bioprocess broth, resulting in gravimetric sedimentation of the heavy SL containing phase, followed
497 by collection and determination of the weight of this phase. However, this crude, viscous SL product
498 typically still contains between 40 to 60 % residual water and other impurities such as fatty acids,
499 salts, residual sugar, glycerol etc. (Roelants et al., 2019), giving rise to a significant overestimation of
500 the SL titres (calculating back to the broth volume). A variation consists of solvent extraction followed
501 by evaporation, which suffers from similar bottlenecks, although often the error will be smaller. In the
502 colorimetric methods, anthrone reacts with carbohydrates, which results in a green product with an
503 absorption maximum at 630 nm. Hence, a rough SL titre estimate can be made by calculating back the
504 obtained carbohydrate value to glucose molecules present in SL molecules, which requires the
505 researchers to make an 'educated guess' of the mean molecular weight of the produced SLs as they are
506 -as mentioned- produced as a mixture. The 'choice' of the molecular weight of SLs in this calculation
507 gives rise to variations in the deduced titre of up to 17 %. Moreover, any residual carbohydrates/sugars
508 (e.g. glucose used as a substrate) in the sample should be considered and should be measured
509 independently and subtracted from the detected 'carbohydrates' (de Bruyn et al., 1968). Such methods
510 can be used for screening purposes but should be avoided to report titres in further scaled-up processes
511 (shake flask and bioreactor scale). Other methods for quantification are more advanced analytical
512 methods such as (U)HPLC-UV, (U)HPLC-ELSD, GC-MS, (U)HPLC-MS^N, ESI-MSⁿ, MALDI-TOF
513 and NMR; but the limitation there is that quite a lot of effort is required to develop a solid and efficient
514 quantification method. Smyth et al. (2014) published standard methods for purification, identification
515 and quantification of glycolipids, which should definitely be considered in future reporting. An
516 adequate method for SL analysis and quantification is the use of (U)HPLC-ELSD in combination with
517 pure standards (>98%). A drawback of this method is that pure standards are not commercially
518 available and should thus be made in-house. Each quantitative SL analysis should be accompanied

519 with a qualitative analysis *e.g.* using MS and/or NMR analysis. This is necessary because some SL
520 congeners share retention times. NMR or MSⁿ analysis is also required to thoroughly characterise the
521 glycolipid mixture produced by newly developed or -discovered SL production strains.

522 The abovementioned aspects should be considered when performing and reporting on process
523 optimisation efforts. Process development typically consists of three interconnected modules:
524 medium- and substrate- optimisation, bioreactor- and downstream processing development.

525 4.1 *Medium- and substrate development and -optimisation*

526 Medium development and -optimisation are of utmost importance when developing novel
527 bioprocesses. The thorough investigation thereof for SLs was often overlooked in the past as the
528 evaluation of different substrates and medium components at varying concentrations and the
529 examination of potential interactions requires many replicates. To decrease labour intensity, a number
530 of ‘high’ throughput screening methods based on deep well plates have been described. Van
531 Renterghem et al., (2019) evaluated the effect of different yeast extracts on SL production with an
532 optimised deep-well method. High throughput screening was combined with Cu(OH)₂ and I₂ assisted
533 detection of, respectively, carbohydrates and sophorolipids (Lin et al., 2019; Zhou et al., 2019).
534 Though such colorimetric methods can be used in combination with a (fractional) factorial or centroid
535 experimental design to perform a cost-effective screening (Jiménez-Peñalver et al., 2018, 2016;
536 Ribeiro et al., 2012b; Rispoli et al., 2010), an accurate final quantification and qualification is still
537 necessary, as discussed above. It is very important to validate a high throughput screening method, *i.e.*
538 it should simulate a larger scale set up (*i.e.* bioreactor scale) allowing the generation of results relevant
539 towards upscaling.

540 As mentioned above, SLs are secondary metabolites, which indicates that the production onset
541 occurs when cells switch from the growth phase to the stationary phase. Therefore, a distinction
542 should be made between base ‘batch’ medium and fed-batch substrates. The former is optimised for
543 growth and stimulate production onset while the latter is fed to optimise SL production in the
544 stationary phase.

545 4.1.1 Base ‘batch’ medium for biomass generation and SL production onset

546 Though SL production is almost exclusively occurring in the stationary phase, increased biomass
547 levels give rise to increased SL productivity (until a plateau is reached (Gao et al., 2013)). A cost-
548 efficient growth phase is favoured, as short as possible and resulting in increased biomass at the start
549 of the stationary phase (Ciesielska et al., 2013; Gao et al., 2013). Both scenarios can be accomplished
550 by tailoring the batch medium components.

551 The transition from growth to stationary phase is usually triggered by a limitation of nitrogen
552 and/or phosphorous resources in combination with an excess of carbon, more specifically, a high C/N

553 ratio (Albrecht et al., 1996; Davila et al., 1992). This limitation does not equal an absence, as SL
554 production occurs while nitrogen and phosphorous are still present in sufficient amounts (Ciesielska et
555 al., 2013). Nitrogen limitation remains the most preferred strategy to trigger the stationary phase.

556 The nitrogen source and its relative concentration is therefore highly important as it will
557 greatly influence the efficiency of the growth phase. Although the *Starmerella* clade is known for its
558 limited range of nitrogen sources that can be assimilated (Gonçalves et al., 2020; Shen et al., 2018),
559 several were evaluated: NH₄Cl (Lang et al., 2000), urea (Vedaraman and Venkatesh, 2010), yeast
560 extract (YE) (Casas and Garcia-Ochoa, 1999), NaNO₃ (Konishi et al., 2015), NH₄SO₄ (Ribeiro et al.,
561 2012a), malt extract (Rispoli et al., 2010), peptone extract (Rispoli et al., 2010), soytone (Rispoli et al.,
562 2010), corn steep liquor (Develter and Fleurackers, 2012), ... As there are multiple synergistic effects
563 between medium components and SL producing organisms differ in nutritional requirements, it is
564 difficult to make hard conclusions, but some trends can be observed:

- 565 1) Organic nitrogen sources like YE are predominantly used, as they are also a source of vitamins
566 and metal ions (Casas and Garcia-Ochoa, 1999; Göbbert et al., 1984; Hommel et al., 1994; Lang
567 et al., 2000; Van Renterghem et al., 2019).
- 568 2) The C/N ratio is of utmost importance as a high C/N ratio (8-9) results in a higher specific
569 production but a lower biomass, while a low C/N ratio (3.5-4) results in lower production but
570 higher biomass. A balanced C/N ratio (4.5-5) results in an optimal SL and biomass production
571 (Van Renterghem et al., 2019).
- 572 3) Industrial scale sophorolipid production has made reference to the use of corn steep liquor
573 (Develter, D.; Fleurackers 2012); while it has been noticed that this results in lower biomass and
574 lower SL production in comparison to YE in a deep well scale screening experiment (Van
575 Renterghem *et al.* 2019).

576 4.1.2 Metals

577 In addition to carbon (C) and nitrogen (N) resources, some metal ions have been described to have a
578 profound effect on *S. bombicola*'s growth and/or SL production. The influence of several ions (Mg²⁺,
579 Zn²⁺, Fe³⁺, Fe²⁺, Ni²⁺, Mn²⁺, Cu²⁺, Co²⁺) as sulphate salt additives was investigated. MgSO₄ favours the
580 production of lactonic SLs, Fe²⁺ favours acidic SL production and addition of Cu²⁺ increased the total
581 SL content 2.16 fold (Chen et al., 2014). Rispoli et al. (2010) on the other hand observed a negative
582 effect of MgCl₂ on SL production. These different results probably originate from two reasons: the use
583 of two different counterions and the use of another base medium (glucose, YE and ureum (Rispoli et
584 al., 2010) vs glucose, YE, KH₂PO₄, Na₂HPO₄ and oleic acid (Chen et al., 2014)). It is thus advised to
585 the effect of the counterions and the interaction effect between the substrates.

586 4.1.3 Fed batch substrates

587 Besides medium components and cofactors necessary to produce biomass and to induce SL
588 production, also the substrates which are used (in)directly towards conversion into SLs are important
589 factors of the medium. Typically, a combination of hydrophilic and aliphatic carbon substrates is fed
590 towards increasing SL productivity. While the SL producers of the *Starmerella* clade have a limited
591 range of hydrophilic carbon molecules they can metabolise (on average 10 ± 5 out of 47 tested carbon
592 sources), *Pseudohyphozyma bogoriensis* can assimilate a wider variety of hydrophilic molecules (33
593 out of 47 tested carbon sources), including α,α -trehalose, D-xylose and D-arabinose (Centraal bureau
594 voor Schimmelculturen, 2020; Kurtzman, 2012; Pimentel et al., 2005). SL producing organisms can
595 typically also assimilate a high variety of aliphatic substrates: oils (Daverey and Pakshirajan, 2009),
596 fatty acids (Li et al., 2016), alkanes (Ito et al., 1980), fatty alcohols (Brakemeier et al., 1998a; Van
597 Renterghem et al., 2018), fatty acid methyl esters (Konishi et al., 2018), Most literature describing
598 SL production processes focusses on glucose (Lang et al., 2000; Solaiman et al., 2015), sucrose
599 (Klekner et al., 1991) or glycerol (Konishi et al., 2017; Lin et al., 2019) as hydrophilic substrates.
600 While for the aliphatic substrate, $C_{18:1}$ containing sources such as rapeseed oil, high oleic sunflower oil
601 (HOSO) or oleic acid are preferred (Cavalero and Cooper, 2003; Van Bogaert et al., 2011; Van
602 Renterghem et al., 2018; Zhang et al., 2011). The substrate selection will depend on a trade-off
603 between the substrate cost and the associated SL productivity. Some cheaper substrates (e.g. waste
604 streams) can result in a dramatic decrease in SL productivities and therefore an associated higher
605 production cost, although the substrate cost is substantially lower. This is an important aspect to keep
606 in mind, certainly so because substrate cost only becomes an important denominator of the production
607 costs at the very large industrial scale (multiple 1000 tonnes level) (Roelants et al., 2018) and the use
608 of waste substrates might complicate registration and commercialisation of derived SLs (certainly for
609 some markets).

610 4.1.4 Second generation resources and waste streams

611 Baccile et al. (2017a) observed in a life cycle analysis that glucose and rapeseed oil contributed for
612 41% and 47% to the total environmental impact of an investigated SL production process,
613 respectively, in total representing 87% of the impact. Moreover, these first generation substrates are
614 resources which compete with the food industry (plant oil, starch derived food grade glucose, sucrose,
615 etc.). Thus, to increase sustainability, to reduce the ecological footprint, to avoid the competition with
616 food production and to promote a circular economy, the use of 2nd generation substrates and waste
617 streams is under investigation. Waste streams are also cheaper than 1st generation substrates, which do
618 account for 10%-30% of the total cost of a bioprocess (Mnif and Ghribi, 2016). Potential pretreatment
619 costs, higher downstream processing (DSP) costs (medium containing more impurities), quality issues
620 towards registration of derived products and lower productivities hampers the breakthrough of 2nd
621 generation economic viable industrial processes (Bhangale et al., 2014; Shah et al., 2017; Takahashi et

622 al., 2011). Further strain and process engineering towards the more efficient use of these types of
623 substrates and the robustness against medium inhibitors is necessary to increase the economic
624 competitiveness of these processes. Recently, an economical viable SL production process was
625 established using hydrolysed food waste as batch medium combined with fed glucose and oleic acid
626 (Kaur et al., 2019; Wang et al., 2020a). Comprehensive and complete overviews of the utilisation of
627 2nd generation resources and waste streams towards SL production were recently provided by Jiménez-
628 Peñalver et al., (2019), Wang et al., (2019) and Ma et al., (2020) and the reader is referred to these
629 reviews for more in depth information.

630 4.2 *Bioreactor optimisation*

631 Generally, three bioreactor methods have been evaluated for SL production: fed-batch production,
632 continuous production with *in situ* product removal and solid-state production.

633 4.2.1 Fed-Batch production

634 Fed-batch production is the most applied towards SL production. The process starts typically with a \pm
635 2 days cell-growth phase resulting in biomass production with a typical end-titre of 7 to 30 g·L⁻¹ cell
636 dry weight (CDW). Subsequently, an 8-14 days SL production phase is conducted where hydrophilic
637 and aliphatic substrates are fed semi- or non-continuously. Common mean SL productivities are in the
638 range of 0.51-2.1 g·L⁻¹·h⁻¹ (Roelants et al., 2019). Gao et al. (2013) described a high cell density
639 process which reached 80 g·L⁻¹ CDW and a mean volumetric productivity of 3.7 g·L⁻¹·h⁻¹. However,
640 production levels could not be maintained due to the rapidly increasing required working volume
641 caused by the high production rate requiring fast feeding of the substrates (Gao et al., 2013). When SL
642 concentrations increase above about 200 g·L⁻¹ (typically after 8-14 days), the viscosity of the broth
643 increases and oxygen transfer decreases, which negatively impacts SL productivity (Zhang et al.,
644 2018). Examples of fed-batch processes are described in detail by Roelants et al. (Roelants et al.,
645 2019), Van Bogaert and Soetaert (Van Bogaert and Soetaert, 2011), Jiménez-Peñalver et al. (Jiménez-
646 Peñalver et al., 2019) and Wang et al. (Wang et al., 2019)

647 4.2.2 *In situ* product removal

648 Fed-batch production is hindered by maximum volumetric capacity of fed substrates at high
649 production rates and reduced productivities caused by limited oxygen transfer over time. These issues
650 urged researchers to develop integrated processes such as *in situ* product removal (ISPR) coupled to a
651 fed-batch or (semi-) continuous bioprocess set-up. Such systems are also developed aiming to decrease
652 down-time and simplify DSP. There are different ISPR methods possible depending on the specific SL
653 congener produced (figure 4). If the product has low water solubility *such as* lactonic SLs (Hu and Ju,
654 2001), ISPR can be obtained through a gravimetric settling set-up. Dolman et al. (2017) described a
655 semi-continuous set-up with a 'separator' coupled to the bioreactor for the production of wild type
656 SLs. When favourable separation conditions are met (sufficient SL concentration and ideal rheological

657 conditions), the broth is constantly pumped through the separator, where the SLs precipitate and the
658 broth with a reduced SL content is pumped back into the bioreactor, resulting in a lower viscosity. The
659 latter separation is performed in a cyclical set-up. Depending on the glucose concentration being lower
660 or higher than $50 \text{ g}\cdot\text{L}^{-1}$, the SLs will situate respectively in the bottom or top layer in the separator
661 (figure 4C and 4D). Hence, six cycles were performed using a top layer separator which resulted in a
662 relatively long cultivation time of 1023 h with a mean productivity of $0.61 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$, substrate yield of
663 $0.47 \text{ g}\cdot\text{g}^{-1}$ and a total separation efficiency of 65%. Nevertheless, higher total separation efficiencies
664 were reached in shorter cultivation times. Utilising a top separation set-up amounted to 86 % for a
665 process of 3 cycles and 305 hours. While, a bottom separation set-up resulted in a total separation
666 efficiency of 74% for a process of 4 and 379 hours. The reason for this lower separation efficiency for
667 the longer process time was described by the authors to be due to variation in SL concentration present
668 in the bioreactor before the separation.

669 A similar but batch operated gravimetric ISPR for wild type SLs was described by Liu et al.
670 (2019) (figure 4B). The authors altered the rheological conditions by adjusting the rapeseed oil/SL
671 ratio to 0.08-0.12 by oil addition prior to pumping the broth into a pre-separator to remove excess air.
672 The latter was coupled to a gravimetric separator of which the top layer was pumped to a washing
673 tank where the SLs could settle. The broth was recycled and pumped back into the bioreactor for
674 further production. This procedure was performed three times and a mean productivity of $1.55 \text{ g}\cdot\text{L}\cdot\text{h}^{-1}$,
675 a substrate yield of $0.43 \text{ g}\cdot\text{g}^{-1}$ was achieved. An overall separation efficiency of 91% was reached, but
676 this did not take into account the SL loss in the final separation step upon termination of the
677 bioprocess. The system has the drawback of being operated in batch mode and therefore the process is
678 idle while performing the separation step.

679 Aforementioned ISPR systems provide some clear benefits, such as smaller reactor size
680 required towards the production of a certain SL production volume, which decreases capital
681 expenditures (CAPEX). However, the constant removal of SLs will require adequate feeding regimes
682 of hydrophilic and especially hydrophobic substrates as these are removed together with the SLs. For
683 hydrophilic substrates, this will result only in reduced yields on substrate and thus negatively affect
684 cost-efficiency (substrate lost), while for the hydrophobic substrates, in addition, it is notoriously
685 difficult to separate them from the SLs, which will require further purification steps. This increases
686 costs and negatively impacts the environmental impact of the overall process as determined through
687 life cycle analysis (LCA).

688 To mitigate the hydrophobic substrate loss, Zhang et al. (2018) developed another ISPR
689 design for wild type SLs which utilises dual sieve plates inside the bioreactor coupled to two semi-
690 continuous separation units, one for the separation of SLs from bioreactor broth by applying soybean
691 oil enhancing separation and a second unit to separate the SLs again from the soybean oil (figure 4E).

692 These authors did not mention the separation efficiency of the reported processes. As the working
693 volume of the reactors was also not reported, the separation efficiency cannot be calculated either.
694 Based on the dimensions of the reactor, the separation efficiency can be estimated between 70 % and
695 80%. A mean volumetric SL productivity of $1.59 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and a substrate yield of $0.59 \text{ g}\cdot\text{g}^{-1}$ was
696 reached with a final titre of $477 \text{ g}\cdot\text{L}^{-1}$ by using *Candida albicans* O-13-1. As mentioned before, *C.*
697 *albicans* has never been described in literature to produce SLs, so this study most probably concerns *S.*
698 *bombicola*. Moreover, the SL quantification was done by using the anthrone method, which can be
699 inaccurate, as described in the introduction of section 4. Moreover, the specific role of the sieve plates
700 was not well explained, and it is thus not clear if these sieve plates actually represent a benefit
701 compared to the more straightforward ISPR systems described above and below or even to a regular
702 bioreactor system without sieve plates applied towards a cyclic process of production and
703 sedimentation. Moreover, the separation set up is quite complex and would not be easily scalable.
704 Also, no information about the residual soybean oil in the SL fraction was reported, which is expected
705 to be substantial when using soybean oil as a process aid to enhance separation of SLs from process
706 broth.

707 *Figure 4: Schematic representation of ISPR production processes reported for SLs. A) Process by (Wang et al.,*
708 *2020a), B) Froth separation by Liu et al. (2019), Gravity separation by Dolman et al. (2017) with C) SL in upper*
709 *phase & D) SL in lower phase, E) Dual sieve-plates and dual ventilation pipes bioreactor by Zhang et al. (2018)*
710 *F) Cell recycle process for bolaform sophorolipids (Roelants et al., 2018). I) batch separator, II) continuous*
711 *separator, III) continuous tangential microfilter (0.2 μm) IV) washing tank. Blue lines represent feed, red lines*
712 *sophorolipid depleted flows and orange lines sophorolipid enriched flows.*

713 Recently, Wang et al. (2020a) described another ISPR process which uses a batch separator
714 process optimised for low hydrophobic substrate loss (figure 4A). Though having the same drawback
715 as the system of Liu et al. (2019), being that the system is operated in batch mode, a mean volumetric
716 productivity of $2.39 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ and a substrate yield of $0.73 \text{ g}\cdot\text{g}^{-1}$ over 480h of production time was
717 achieved while utilising a restaurant left over waste stream as batch medium fed with glucose and
718 oleic acid in the fed batch phase. The overall separation efficiency over 6 cycles and final separation
719 step amounted to 93%. A techno-economic analysis was performed for a full-scale SL production
720 plant based on this process. Multiple scenarios indicate lower minimum selling prices in comparison
721 to the market price ($13.87 \text{ euro}\cdot\text{kg}^{-1}$ vs. $21.62 \text{ euro}\cdot\text{kg}^{-1}$, respectively) (Wang et al., 2020b).

722 A different type of ISPR was developed for bolaform SLs, which are, in contrast to lactonic
723 SLs, highly water soluble (Van Renterghem et al., 2018). This process couples a $0.2 \mu\text{m}$ filter to the
724 bioreactor and a continuous microfiltration is performed where broth enriched in cells is pumped back
725 to the bioreactor, whereas filtrate containing medium components, substrates and bolaform SLs is
726 constantly removed (figure 4F). A mean volumetric productivity of $0.63 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ was reached and
727 could be maintained for 240 hours, after which it slowly declined (Roelants et al., 2018). Like for the

728 set up described by Dolman et al. (2017), it can be remarked that the substrates are ‘lost’ in the filtrate.
729 However, a subsequent purification step on the filtrate e.g. ultrafiltration can be imagined where the
730 bolaform SLs are separated from the remaining medium components, which can then be pumped back
731 into the bioreactor (Roelants et al., 2016). The way the set up was now described a separation
732 efficiency of about 100% was achieved as the concentration bolaform SLs pumped out of the reactor
733 was the same as the concentration bolaform SLs in the bioreactor. It should be noted that such set up
734 can only be applied with compounds with high water solubility, while the set ups described above for
735 lactonic SLs on the contrary can only be applied with compounds with low water solubility.

736 ISPR proved to be an efficient and economical viable technique to increase SL productivity at
737 lab-scale. However, higher operational expenses can be predicted because ISPR systems in general
738 require more follow-up (Palme et al., 2010). Therefore, an economic analysis should be performed to
739 determine if an ISPR design has an economical beneficial effect. Also, attention should be given to the
740 feasibility of scaling-up ISPR procedures which can be particularly challenging. However, two
741 companies are currently applying ISPR techniques for SL production at the larger scale, which
742 confirms the industrial relevance. Holiform (Manchester, UK) communicated that they started with the
743 construction of a pilot plant (300L) based on the ISPR set-up described by Dolman et al. (2017). Also,
744 Locus (Solon, Ohio) is utilizing a similar ISPR technique for SLs production (Sean et al., 2020). They
745 filed a patent that describes a large-scale apparatus that includes a bioreactor vessel and a directly
746 connected collector vessel. The main challenges associated with these systems are residual
747 hydrophobic substrates remaining together with the collected SLs as they are required for optimal SL
748 production.

749 4.2.3 Solid state production

750 Solid state production is a process that takes place in a solid matrix in absence or near absence of free
751 water (Thomas et al., 2013). The advantage is that (cheaper) solid substrates can be used while no
752 foam control is necessary (Jiménez-Peñalver et al., 2016). Jiménez-Peñalver et al. (2018) used a foam
753 of polyurethane with sugar beet molasses and stearic acid (1.17:1 w/w) as substrates to obtain a yield
754 of 0.211 g SLs per g of substrates. The use of solid waste streams in a solid state production set-up has
755 also been evaluated, e.g. mango kernel (Parekh et al., n.d.), safflower cake (Nooman et al., 2017),
756 motor oil waste and sunflower oil cake (Rashad et al., 2014). The effect of mixing steps of a solid state
757 consisting out of winterisation oil cake and sugar beet molasses was profound and resulted in an
758 increase from 0.179 g SL/g dry mass (DM) to 0.235 g SL/g DM. A correlation was observed between
759 the oxygen uptake rate (OUR), cumulative oxygen uptake rate (COC) and the SL substrate yield
760 (Jiménez-Peñalver et al., 2016).

761 Solid state production can reach industrial standards for waste stream processing but needs to
762 resolve its key issues: the lack of heat dissipation, minimal process monitoring and down-stream

763 processing. The latter is currently only possible with solvent extraction methods, which are not
764 preferred, mainly due to environmental reasons (Jiménez-Peñalver et al., 2019).

765 4.3 *Downstream processing optimisation*

766 The downstream processing (DSP) cost for many processes can contribute up to 60-80% of the total
767 production cost, therefore the development of cost-effective DSP processes is of high interest
768 (Fleurackers, 2013). A common lab scale approach for SL purification is solvent extraction with
769 ethanol (Van Bogaert et al., 2016), ethylacetate (Kurtzman et al., 2010) or pentanol (Baccile et al.,
770 2013). These methods are often accompanied by hexane washing steps to remove residual fatty acids
771 (Baccile et al., 2013). Moreover, silica gel chromatography (Gross et al., 2013) methods have been
772 described. However, all above mentioned methods use organic solvents, which is not the preferred
773 method for industrial production of SLs, because it conflicts with the green nature of microbial
774 biosurfactants (Jessop, 2011; Thavasi and Banat, 2018). Therefore, different DSP methods are
775 described, mitigating the use of solvents, *e.g.* the use of filtration techniques (Baccile et al., 2017a;
776 Roelants et al., 2016; Van Renterghem et al., 2018) or separation based on melting at 55°C of SLs
777 followed by a sedimentation, separation and water washing (Roelants et al., 2016). A possible
778 subsequent phase can be the crystallisation and drying of SLs crystals, but this can result in increased
779 costs, so a trade-off should be made between purity and cost. More information concerning DSP
780 methods is described by Thavasi and Banat (2018).

781 It should be considered that when using waste streams as substrates, the USP (upstream
782 processing) and DSP can become more elaborate. There is a higher market tolerance for increased
783 impurities for low-end applications (*e.g.* detergents) compared to high-end applications (*e.g.* food,
784 nanotechnology). The final product of a WT SL mixture production process is either a SL syrup (78%
785 dry matter) or SL crystals (97% dry matter) (Roelants et al., 2016; Wang et al., 2020b). A techno-
786 economical study determined the CAPEX of a crystalliser and a freeze-dryer, both necessary to create
787 SL crystals, equals 68.5 % of the CAPEX cost of the bioreactor (Wang et al., 2020b). Furthermore,
788 freeze-dryers are known to have a high energy demand. In contrast, SL crystals are more stable and
789 have a longer shelf life in comparison to a syrup (Van Bogaert and Soetaert, 2015). Moreover, the
790 impact of transportation of SL crystals is less as it contains less ‘dead weight’, *i.e.* water. Therefore, an
791 economical and environmental trade-off should be made while taking the aimed final product in mind.

792 **5 Multipurpose compounds with a broad application potential**

793 The applications of SLs have extensively been reviewed elsewhere (de Oliveira, 2015; Develter and
794 Laurysen, 2010; Hayes et al., 2019; Santos et al., 2016; Van Bogaert et al., 2007). The aim of this
795 section is to complement these reviews and to focus on recent (2015-2020) and most promising
796 proceedings in terms of SL applications.

797 SLs can be applied for their bio-active properties and/or for their surface-active properties.
798 These properties can vary significantly in function of purity (*e.g.* residual presence of hydrophobic
799 substrate(s)), uniformity (*e.g.* ratio lactonic versus acidic SLs) and formulation matrix (water-, oil- or
800 solvent based), so it is of utmost importance to keep these in mind when evaluating application
801 literature. For example, the reported bioactive properties reported for (wild type) SLs vary a lot. such
802 as anti-microbial properties can reach very diverse values. However, this can clearly be attributed to
803 the fact that the ratio between lactonic and acidic SLs and their acetylation degree in a (wild type) SL
804 mixture depends on the culture conditions and the fact that lactonic and acidic SLs differ in bioactivity
805 (Ciesielska et al., 2016). Information on purity and SL congener ratios should thus be included in
806 future publications to avoid further confusion on the potential application areas of ‘sophorolipids’
807 (Twigg et al., 2020).

808 As many applications of SLs relate to the self-assembly properties of SLs in water, the latter
809 will be discussed first, followed by the literature review of specific SL application areas. These areas
810 not surprisingly are to a great extent in line with the surfactant market and the use of classic
811 surfactants over a range of markets with varying market volumes. These include, from large to
812 smallest: household detergents, personal care, textile, industrial and institutional (I&I) cleaning,
813 elastomers and plastics, oilfield chemicals, food & beverages, crop protection and smaller scale use
814 markets such as pharma, construction, paints and inks, etc.

815 5.1 *Self-assembly properties of sophorolipids*

816 5.1.1 General principles of surfactant self-assembly in solution

817 Surfactant self-assembly in solution generally refers to an equilibrium process driven by the so-called
818 hydrophobic effect, involving non-specific interactions between aliphatic chains of surfactants. This
819 attractive force is counterbalanced by a number of repulsive contributions to the free energy,
820 including, but not limited to, steric, electrostatic and interfacial components. The first two
821 contributions occur at the level of the hydrophilic headgroup while the latter occurs at the micelle-
822 water interface (Israelachvili et al., 1976; Tanford, 1973). The simplest self-assembled morphology of
823 a surfactant in solution is a micelle, spheroidal object forming above a critical concentration of the
824 surfactant in solution. The shape of micelles can evolve towards cylinders, vesicles or lamellae,
825 according to concentration and in relationship to the shape of the surfactant’s molecule (Israelachvili et
826 al., 1976). The critical micelle concentration (cmc) in water is commonly measured for all surfactants
827 (Holmberg et al., 2002) and cmc values for sophorolipids were reported before (Daverey and
828 Pakshirajan, 2010; Hirata et al., 2009; Kim et al., 2005; Mnif et al., 2018; Solaiman et al., 2004). On
829 the other hand, knowledge of the molecular structure (tail length and volume, headgroup surface area)
830 helps understanding the shape of the self-assembled aggregates in solution. This last aspect has been
831 rationalised long ago in the so-called critical packing parameter (PP): the lower the PP, the stronger

832 tendency for the surfactant to assemble into spheroidal micelles; the higher the PP, the stronger its
833 tendency to assemble into flat bilayer membranes (Holmberg et al., 2002; Israelachvili et al., 1976). It
834 goes without saying that, although the PP approach has been verified over the years, it still constitutes
835 an oversimplified way to understand self-assembly and it has been shown to fail in many cases, due to
836 which more recent general theories have been proposed (Bergström, 2000a, 2000b). We have recently
837 revised the PP theory and discussed it within the context of biosurfactants' self-assembly (Baccile et
838 al., 2021b).

839 5.1.2 Self-assembly of sophorolipids in solution

840 Sophorolipids, compared to classical head-tail chemical surfactants, have a triple level of complexity:
841 1) structural., because they are asymmetric bolas, instead of common head-tail, amphiphiles; 2)
842 chemical, because they contain chemically-reactive and physicochemically-active groups (C=C,
843 carboxylic acid); 3) compositional, because sophorolipids are rarely pure molecules but generally a
844 mixtures of similar congeners with different properties. Speaking of sophorolipid self-assembly is then
845 highly imprecise, and one should state which SL are concerned and the pH at which self-assembly is
846 studied. In this regard, it is not surprising that the critical micelle concentration (cmc) of sophorolipids
847 reported over the years in the literature vary from 6 to about 700 mg/mL (Daverey and Pakshirajan,
848 2010; Hirata et al., 2009; Kim et al., 2005; Mnif et al., 2018; Solaiman et al., 2004). Table 2
849 summarises the up-to-date work published on the solution (water) self-assembly properties
850 (concentrations below 10 wt%) of a number of sophorolipids and their derivatives and recently
851 reviewed in Ref. (Baccile et al., 2021b). We present below a short comment for each specific phase.
852 Please note that appropriate references are given in Table 2.

853
854 *Micellar phase (L1)*. This phase is by far the most representative for a large number of
855 sophorolipids, except lactonic SLs, at concentration below 10 wt% (Table 2, *Micellar* column). The
856 L1 phase is systematically observed for the charged, COO^- or NH_3^+ , forms of SLs at pH respectively
857 above and below neutrality. This can be explained by the repulsive electrostatic contribution to the
858 intermolecular forces as well as by the bulky sophorose headgroup. For specific SL derivatives (e.g.,
859 alkynyl, peracetylated porphyrin), the micellar phase is controlled by temperature, instead. The L1
860 phase is found for a broad pH range, from acidic to basic, for both the acidic (R-COOH) and aminyl
861 (R-NH₂) forms of C18:1-*cis* nonacetylated SLs, the former being the most common form of SLs,
862 easily obtained by hydrolysis of acetylated lactonic SLs. It cannot be excluded that stabilization of the
863 micellar phase in a broad pH range may be due to the presence of spurious amounts (< 10%) of SL
864 congeners, naturally present in the raw mixture and hard to remove (Dhasaiyan et al., 2017). The
865 structure of SL micelles has been studied in depth using a combination of small angle X-ray scattering
866 (SAXS) and numerical modelling. The localization of the COO^- groups has been probed through the
867 distribution of their counterions via anomalous SAXS experiments while the size and shape of the

868 hydrophobic core through contrast-matched small angle neutron scattering. The bolaform nature of
869 SLs produces a spheroidal (prolate ellipsoid) micelle with a small hydrophobic core and an
870 asymmetric hydrophilic shell (*coffee-bean* like model) (Manet et al., 2015). The micellar phase has
871 also been reported for a number of asymmetrical, symmetrical, divalent and Y-shaped SL derivatives
872 (N. Baccile et al., 2019a).

873

874 Micelles of C18:1-*cis* nonacetylated SLs (COOH or NH₂ end-groups) are neutral or charged
875 according to the ionization state of the end-group and, in this regard, they can interact with other
876 molecular species, like polyelectrolytes (Ben Messaoud et al., 2018; Seyrig et al., 2020) or proteins
877 and enzymes (Andersen et al., 2016; Madsen et al., 2017). Strong electrostatically-driven SLs-
878 polyelectrolyte complexes present interesting possibilities in emulsion stabilization through soft
879 colloids, (Laquerbe et al., 2021) whereas acidic SLs were shown to be poor emulsion stabilizers. In
880 this regard, acidic-lactonic SL mixtures and alkyl-modified SLs were shown to have better emulsion
881 stabilization properties than acidic SLs (Koh et al., 2017a, 2016; Koh and Gross, 2016a, 2016b). On
882 the contrary, interactions between SLs and proteins and enzymes were shown to be relatively mild, if
883 compared to other ionic surfactants, thus limiting their denaturation (Andersen et al., 2016; Madsen et
884 al., 2017).

885

886 *Vesicle phase.* This phase is relatively rare for SLs, probably due to their bulky sophorose
887 headgroup. Multilamellar vesicles (MLV) have been reported mainly for behenic (C22:0₁₃) and
888 linolenic (C18:3-*cis*) derivatives of SLs at neutral/acidic pH (Table 2, *Vesicle* column) while single
889 unilamellar vesicles (SUV) have been reported for lactonic SLs.

890

891 *Lamellar phase.* This phase (Table 2, *Lamellar* column) is particularly rare, and it has been
892 most likely observed as a precipitate for behenic derivative of SL (C22:0₁₃) at acidic pH, upon full
893 protonation of the COOH group.

894

895 *Columnar phase.* This unique phase has been reported for hydroxylated porphyrin dimers of
896 SLs, and it is strictly related to the presence of the porphyrin group, but also to the presence of residual
897 OH groups (Table 2, *Columnar* column). Such compounds could serve to develop new biocompatible
898 exciton-coupled chromophores.

899

900 *Fibre phase.* This phase, in the morphology of twisted ribbons, has been surprisingly observed
901 for a large number of SL derivatives, thus demonstrating the complexity of the self-assembly process
902 of this compound. Initially reported for deacetylated C18:1-*cis* sophorolipids under acidic conditions
903 (Zhou et al., 2004) (Dhasaiyan et al., 2013), this result was questioned years later by others, showing
904 that such compound rather forms a micellar phase (Baccile et al., 2016a, 2012, 2011; Penfold et al.,

905 2011). The presence of a mixture of congeners (one of which forms twisted ribbons of its own) in the
906 analysed samples partially explained such discrepancy (Dhasaiyan et al., 2017). The fibre phase has
907 been otherwise observed for the neutral form of C18:0 SL derivatives, may they have an end-COOH
908 (acidic pH) or -NH₂ (basic pH) group, but also for C18:1 alkynyl SLs, C18:1-*trans* (acidic pH), C16:0
909 (acidic pH) SLs bolaform, di-sophorose, sophorosides and SL derivatives. (Table 2, *Fibre* column).
910 Interestingly, the C18:1-*trans* SL derivative in its ionic form at basic pH also shows the formation of a
911 fibre phase in the order of hours.

912 The reason according to which this phase is so often observed for a broad set of SL derivatives
913 is still unclear. It does not seem to be related to a specificity of the sophorose headgroup itself, as
914 cellobioselipids display a similar phase (Baccile et al., 2016b). It could be related to the all-*trans*
915 configuration of the SL fatty acid backbone, but the C18:1-*cis* alkynyl derivative also shows this
916 phase. It could be related to the chiral centres of the sophorose headgroup, but no specific
917 enantiomeric excess could be detected in the fibre twist (Cuvier et al., 2015a). Following the
918 hypotheses formulated by others (Barclay et al., 2014), fibre formation could be a kinetic phenomenon
919 related to the poor solubility in water of the above-cited molecules and in combination with the
920 bulkiness of the sophorose headgroup, which could drive the twist through steric hindrance and limit
921 later crystal growth during the nucleation and growth of the fibres. The kinetic aspect seems to be
922 justified by the fact that the C18:0 SL derivative forms flat crystals upon dispersion in water
923 (Dhasaiyan et al., 2013) but twisted fibres through a fast pH-jump process (Cuvier et al., 2014). In the
924 latter, fibres can be homogeneous or in the shape of spherulites, according to the rate of pH variation
925 (Ben Messaoud et al., 2019), whereas the presence of salt seems to play an important role in the
926 fibrillation process (Ben Messaoud et al., 2019; Cuvier et al., 2015a). It goes without saying that
927 fibrillation of SLs should be studied further for a better understanding.

928 Despite the above, homogeneous fibrillation induces hydrogel formation at concentrations as
929 low as 1 wt% of SL in water (Baccile et al., 2021a, 2018; Ben Messaoud et al., 2019). Controlled
930 variation of pH and temperature directly controls the nucleation and growth of the SL fibres, thus
931 providing not only pH-responsive, but also tough, hydrogels, with elastic modulus as high as 10 kPa,
932 thus paving the way to the development of biosurfactant-based soft materials (Baccile et al., 2019b).

933
934 *Other (minor) phases.* Platelet aggregates or ill-defined phases, mainly detected in SAXS
935 profiles and in cryogenic transmission electron microscopy, are often observed for the ionic form of
936 SLs. They generally involve a small fraction of the compound and to date, neither their origins nor
937 their full control are understood. In some occasions, such phases result in a flocculate visible with bare
938 eye.

939
940 In summary, SLs display a rich phase behaviour, which depends on their structure,
941 physicochemical conditions in solution and purity. The take-home message is that nonacetylated

942 acidic C18:1-*cis* SL, the most accessible compound through alkaline hydrolysis of a raw acidic-
943 lactonic SL mixture, is water soluble and mainly forms a micellar phase in a broad pH range, and of
944 which the surface charge (neutral/negative) depends on pH. This compound is then interesting to
945 develop further formulations with other surfactants and (bio)macromolecules. The fibre phase, on the
946 contrary, is very interesting to develop strong hydrogels and it can be mainly obtained from saturated
947 derivatives of SL under acidic conditions.

948

949 *Table 2: Self-assembled structures of sophorolipids and derivatives reported at room temperature*
950 *(unless otherwise stated). SUV: single unilamellar vesicles; MLV: multilamellar vesicles. #time>1h*
951 *(minor fraction) ##: 1i, 1h, 1f, 1k, 1j, 1l, 1g, 2g (Ammonium- and amine oxide derivatives of SL) and*
952 *3b, 3c, 3d, 4d, 5c, 5d, 6a, 6d (Symmetrical amine SL derivatives) in Ref. (Baccile et al., 2019a) as*
953 *neutral form at basic pH.*

954

955 5.2 *Cleaning/washing*

956 The biggest part of the annual production volume of surfactants (46.3 %) is produced for household
957 detergents (Ranji et al., 2019). Sophorolipids can be used as readily biodegradable, non-toxic, bio-
958 based, non-irritable, ‘green’ formulation agents in detergents and cleaning applications. Research
959 papers and patents describe the use of SLs in hard surface cleaners (Develter et al., 2003; Develter and
960 Fleurackers, 2007; Karsten et al., 2010); anti-scaling, rinse aid, (low-)foaming, wetting, degreasing
961 agents (Hillion et al., 1995; Van Renterghem et al., 2018; Xiaoming et al., 2005) and biosolubilisers
962 (Ernenwein et al., 2013). Besides for their purely physicochemical traits, SLs have been extensively
963 examined for their antimicrobial, antifungal and antiviral properties for disinfectant and/or germicide
964 purposes in cleaning formulations for the medical, food, agricultural and cosmetic industry. Besides
965 direct inhibition of micro-organisms, SLs have been shown to inhibit biofilm formation and disrupt
966 biofilms. Unlike in other application fields, SLs have been applied in B2C products like cleaning
967 products for some years and the Belgian company Ecover (Malle, Belgium) was one of the pioneers.
968 Since the B2B commercialisation of SLs under the name of Rewoferm®SL by Evonik (Essen,
969 Germany), a product honoured with the SEPAWA Innovation Award 2016 for its excellent ecological
970 profile, the application field is expected to open up for more B2C companies. Future commitments in
971 this context should include the further elucidation of the formulative potential of these compounds and
972 the continuous efforts to evaluate for functioning bioactive formulations.

973 5.3 *Personal care and cosmetics*

974 The personal care and cosmetics surfactants market segment is the second largest segment of the
975 surfactant market after detergents, corresponding to a yearly production/use of about 3 million tonnes,
976 and is also the fastest-growing segment of the surfactants market and projected to register a CAGR of
977 5.5%, in terms of volume, between 2016 and 2021 (Markets and markets, 2016). Because this is a very

978 consumer receptive market, it has been clearly redirecting itself the past 5 to 10 years towards more
979 sustainable, green, bio-based and mild products which also do not contribute to deforestation, a clear
980 issue in a market using a lot of palm oil derived products. Due to higher acceptable costs, this is an
981 ideal entry market for new types of (typically more expensive) bio-based surfactants such as SLs.
982 Already in the late 80's the first reports on the use of SLs in such products were published, which
983 ultimately resulted in the commercialisation of SLs in personal care products in the early 2000's. For
984 example, the antibacterial and sebum control agent Sopholiance[®] used in deodorants (Soliance, now
985 Givaudan (Vernier, Switzerland)) or the Sopholin Acne Soap (MG Intobio Co LTD, Icheon, Republic
986 of Korea) both make use of the bacteriostatic and bactericidal properties of SLs to fight odour-, acne-
987 and/or infection inducing bacteria such as *Bacillus subtilis*, *Staphylococcus epidermidis*,
988 *Staphylococcus aureus*, *Streptococcus faecium*, *Propionibacterium acnes* and *Corynebacterium*
989 *xerosis* (Lang and Wagner, 1993; Yatim et al., 2010) or fungi such as *Candida albicans* and
990 *Trichophyton* spp. (Haque et al., 2017; Sanada et al., 2014; Sen et al., 2017). The use of sophorolipids
991 in cosmetic and dermatological compositions was already patented in 1994 (Hillion et al., 1994).
992 Patents describing the use as a mild, foaming detergent and the use in hair and skin cleaning
993 compositions are assigned to, respectively, Unilever (London, United Kingdom) (Cox et al., 2011) and
994 Evonik (Essen, Germany) (Allef et al., 2012). Their use in sulphate-free cleansing agents for their
995 prebiotic activity is also patented (Heike et al., 2016). However, according to publications and other
996 granted patents, interesting properties include more than only formulation aiding or bacterial control
997 agents, as they have been suggested as cellulite reducers through leptin synthesis stimulation in
998 adipocytes (Pellicier and Andre, 2004), and as skin penetration or transdermal release enhancers for
999 active ingredients such as mogroside V (Imura et al., 2014), bovine lactoferrin (Ishii et al., 2012;
1000 Matsumiya et al., 2017) and lignans (Naik et al., 2019). Other patents describe their use to provide free
1001 radical formation inhibiting activity, elastase inhibiting activity and anti-inflammatory activity (Hillion
1002 et al., 1995) or as activator of macrophages, as fibrinolytic agent, as healing agent, as desquamation
1003 agent and as depigmentation agent (Maingault, 1996). The further expansion of the use of SLs in
1004 cosmetic- and personal care products would allow their price to drop, which will result in turn in even
1005 more opportunities and eventually possibly in the mainstream use of SLs and other (microbial)
1006 biosurfactants in personal care and other products, as 'cost' is one of the main bottlenecks.

1007 5.4 *Oilfield chemicals and microbial enhanced oil recovery*

1008 Due to their amphiphilic properties, SLs are able to adsorb at hydrophobic surfaces such as crude oil,
1009 resulting in a reduced surface- and interphase tension, increasing specific surface area and reducing
1010 viscosity (de Oliveira, 2015). These traits can be used in so-called microbial enhanced oil recovery
1011 (MEOR) in which residual oil, accounting for approximately 50% of the oil reserves after primary and
1012 secondary extraction, can be partially recovered from oil wells (Geetha et al., 2018). For *in-situ*
1013 MEOR microorganisms or nutrients are injected into the oil well, while *ex-situ* MEOR uses a purified

1014 product for extraction purposes, both circumventing the use of traditional non-biodegradable and
1015 sometimes unstable chemical counterparts. Although interesting results were seen in core-flooding
1016 experiments in which up to 27.27% of the residual oil was recovered through the use of SLs (Elshafie
1017 et al., 2015), only limited patents or publications are available describing the use thereof for such
1018 applications (Duran 2015; Koral, Weers and Campbell 2014; Elshafie *et al.* 2015; Gunawan,
1019 Vorderbruggen and Armstrong 2017). The need for facultative (an)aerobic bacteria and tricky growth
1020 control for *in-situ* MEOR and the high cost of SLs in the case of *ex-situ* MEOR impedes the
1021 elaboration of SL-MEOR, but also for microbial biosurfactants in general. Another challenge is the
1022 execution of field-scale experiments, which sometimes gives rise to deviating results compared to lab-
1023 scale experiments (Geetha et al., 2018). As long as the scale of industrial SL production remains
1024 relatively small and prices thus remain high, utilisation for high volume, bulk commodities such as
1025 MEOR will not be possible. Geetha, Banat, and Joshi (2018) have summarised the status of the use of
1026 biosurfactants in oil recovery in a very concise way, including issued patents on the matter. Oilfield
1027 chemicals in general represent a market share of about 1 million tonnes or about 5% of the total
1028 surfactant volumes produced (Markets and markets 2016). Decreasing the cost of raw materials, often
1029 an important factor governing the price of the product on the very large industrial scale, by using
1030 nutrient rich industrial wastes as displayed by Joshi et al. (2019) or (Wang et al., 2020a) could be an
1031 important step to increase the economic viability of these applications at least at large industrial scale
1032 as discussed in section 7 (Felse et al., 2007; Roelants et al., 2018). Nevertheless, the use of microbial
1033 biosurfactants, which have amongst others been created as an alternative to petroleum-based
1034 surfactants, in the petroleum industry seems contradictory and should be critically considered.

1035 5.5 *Food and beverages*

1036 The food & beverage industry covers meats, processed foods, beverages, dairy products, baked goods,
1037 candy, snack foods, frozen foods, and fats & oils. Surfactants are used in this industry, with a total
1038 annual market of about 0.9 million tonnes (Markets and markets, 2016), for different purposes linked
1039 with both physicochemical and anti-microbial properties. They have always been an important
1040 formulation component of foodstuffs because of their stabilising, anti-adhesive, emulsion forming or -
1041 breaking and foam forming activity (Kralova and Sjoblom, 2009; Sharma, 2016; Tadros, 2013). At
1042 present, SLs have not yet found their way into the food sector, however, due to their non-toxic nature
1043 (Develter and Laurysen, 2010), their green image and production from renewable resources such as
1044 glucose and sunflower oil, SLs are highly compatible with foodstuffs and thus increasingly gain
1045 interest in this field (Campos et al., 2013). SLs are able to form oil/water (O/W) emulsions with a
1046 range of hydrocarbon and triglyceride oils, suggesting their food applicability (Gross et al., 2013; Koh
1047 et al., 2017b; Sałek and Euston, 2019). Xue et al. (2013) demonstrated that the stabilisation of O/W
1048 emulsions containing structured lipids using SLs was equally effective as compared to Polysorbate 20,
1049 a commonly used food emulsifier. Other studies determined the interfacial surface tension of a series

1050 of alkyl esters of SLs added to mixtures of paraffin and water, almond oil and water and lemon oil and
1051 water to form O/W emulsions, demonstrating optimal SL-ethyl ester concentrations to stabilise the
1052 emulsions (Koh et al., 2016; Koh and Gross, 2016a, 2016b). Besides these tests based on purely
1053 physicochemical characteristics, a number of studies have demonstrated the antibacterial and/or
1054 antifungal effects of SLs against food spoiling and opportunistic pathogenic microbial species (Baccile
1055 et al., 2019a; Díaz De Rienzo et al., 2016; Olanya et al., 2018; Zhang et al., 2016). The potential
1056 application of SLs as emulsifiers and/or preservatives in food products requires studying the taste-
1057 sensory properties of SLs. According to Ozdener et al. 2019 and Solaiman et al. 2019, the addition of
1058 SLs into foodstuffs could have an effect on taste responses as they could ameliorate bitter tastes in
1059 foods and drugs through t1R3 mediated HBO cell response. As mentioned, implementation of SLs into
1060 food stuffs is not a reality yet. The lack of complete and decisive toxicological studies, and the rather
1061 high prices for SLs account for this stasis. A breakthrough could be their added value *e.g.* their use in
1062 high value food products such as dietary supplements and/or nutraceuticals or functional foods. For
1063 example, acidic SLs have been shown to solubilise and nano-encapsulate curcumin, improving its
1064 water solubility, stability and bioavailability and hence enhancing its therapeutic activity. The
1065 exploitation of the multifunctionality of SLs, combining quality improvement through formulation,
1066 prolonging shelf-life and improving therapeutic properties of high value foodstuffs, reduces the need
1067 for other additives, making its application cheaper, more attractive and worth the investment.

1068 5.6 *Crop protection*

1069 About 0,8 million tonnes of surfactants are used every year for the formulation of agrochemicals that
1070 are used for crop protection (Markets and markets 2016). Surfactants are used here for their dispersing,
1071 suspending, wetting, foaming and penetration properties and also help to improve the shelf life of the
1072 products due to their anti-microbial properties. Also, in this sector the demand for bio-based
1073 surfactants is increasing due to a growing use of environmentally friendly chemicals in agriculture.
1074 One might thus exploit the biodegradable and non-toxic character of SLs and hence the use of SLs has
1075 been quite thoroughly investigated for multiple purposes in this sector. The main advantage of using
1076 SLs here is the reduction or total elimination of the need for (chemical) pesticides and fungicides,
1077 limiting the overall costs while maintaining the pesticide or herbicide emulsifying function (Vaughn et
1078 al., 2014) and increasing production yields (Sieverding, 2015). Through the reduction of the surface
1079 tension of herbicides, SLs will aid in the formation of smaller droplets and have a softening effect on
1080 the plant's crystalline waxes, resulting in improved retention on the leaves and thus lower dissipation.
1081 Also increased foliar uptake was described due to better penetration and increased spread (Schönherr
1082 et al., 2000; Vaughn et al., 2014). When using SLs as adjuvant to the commonly used herbicides
1083 glufosinate ammonium (GLA) and lemongrass oil (LGO), the herbicide Cato[®] (DuPont[™],
1084 Wilmington, USA) and the systemic fungicide Opus[®] (BASF, Ludwigshafen am Rhein, Germany),
1085 increased effects have been described in comparison to the formulation without SLs (Vaughn *et al.*

1086 2014), a finding that has been patented (Evonik, Giessler-Blank *et al.* 2010). Antimicrobial and
1087 antifungal effects for plant pathogenic fungi and bacteria such as *Phytophthora sp.*, *Phytium sp.*,
1088 *Alternaria solani*, *Penicillium chrysogenum* and *Botrytis cinerea* have been described and were linked
1089 to inhibition/reduction of mycelial growth, zoospore lysis and zoospore motility. Also these findings
1090 are protected/described in several patents/patent applications (Gross and Shah, 2005; Haque *et al.*,
1091 2017; Sachdev and Cameotra, 2013; Schofield *et al.*, 2011; SyntheZyme, 2012; Yoo *et al.*, 2005). The
1092 potential use of SLs as a germicide or germicide formulator, linked to reducing microbial
1093 contaminations of opportunistic pathogens, has also been described in agricultural work context or
1094 distribution centres of fruits and vegetables (de Oliveira, 2015). The agricultural industry is, besides
1095 the cleaning and cosmetic industry, already applying wild type SLs into their products. Examples are
1096 the Rewoferm[®] product of Evonik described above and ACS-Sophor[®] of Allied Carbon Solutions
1097 (Tokyo, Japan). The use of SLs in this sector is expected to grow the next few years thanks to their
1098 potential application in all stages of crop production, *e.g.* the use for pre-sowing soil treatment as
1099 green biostimulants for seed germination and growth (de Vasconcelos *et al.*, 2020), treatment of soil
1100 for increased bio-availability of micronutrients (Singh, Glick, and Rathore 2018) and vegetable and
1101 fruit washing (section 5.2).

1102 5.7 Surfactant enhanced (bio)remediation, bioflotation and wastewater treatment

1103 The most important environmental and health risk posing contaminants described in recent literature
1104 can be classified as poorly water-soluble organic pollutants, metals, oil fractions derived from
1105 petroleum and radionuclides (Mao *et al.*, 2015). The readily biodegradable and non-toxic nature of
1106 SLs lends them perfect for remediation techniques in the environment, as current technologies are
1107 often inefficient and contribute to additional pollution due to the intrinsic toxicity and/or the very often
1108 low biodegradability of the compounds used (Arelli *et al.*, 2018). This makes surfactant enhanced
1109 bioremediation (SEB) one of the most examined application topics of SLs in literature. The mode of
1110 action of (microbial) biosurfactants is often not yet fully understood, however almost always includes
1111 the increase of the contaminants' solubility and an increase in the specific surface area of a
1112 contaminant bearing matrix or the hydrophobic liquid phase. Also important, but very dependent on
1113 the structure of the used biosurfactant, is the binding of the contaminant to the biosurfactant, thus
1114 leading to contaminant-biosurfactant complexes, often resulting in inclusion into macromolecular
1115 biosurfactant structures such as micelles (Luna *et al.*, 2016; Mao *et al.*, 2015; Sarubbo *et al.*, 2015).
1116 When contaminated sources are subjected to living organisms during remediation, specific non-toxic
1117 biosurfactants could aid their growth by, amongst others, increasing the bioavailability of the
1118 substrate. However, the latter mechanism is not yet fully proven and probably only accounts for
1119 specific biosurfactant-microorganism interactions (Schippers *et al.*, 2000). For example, SLs have
1120 been shown to increase the maximal degradation rate for the microbial remediation of poly-cyclic
1121 aromatic hydrocarbons (PAHs) like anthracene, fluorene, phenanthrene and pyrene, to which the

1122 solubilisation effect exerted by SLs has repeatedly been attributed (Schippers et al., 2000; Song et al.,
1123 2016). This ‘washing effect’ has also been seen for polychlorinated biphenyls, where the removal
1124 efficiency using SLs exceeded that of traditional surfactants Tween-80 and SDS (Qi et al., 2014).
1125 Lubricating oil contaminated soils have been treated by SLs by Minucelli et al. (2017), but most of the
1126 research has been successfully conducted towards remediation and dispersion of petroleum derived
1127 hydrocarbon fractions such as 2-methylnaphthalene, hexadecane, pristane, (bio)diesel, aviation
1128 kerosene and (light) crude oil phases itself in (sea)water, beach sands and soils (Babaei and Habibi,
1129 2018; Goswami et al., 2020; Kang et al., 2010; Saborimanesh and Mulligan, 2018). The concepts
1130 described above are also seen during biosurfactant or sophorolipid enhanced anaerobic fermentation in
1131 wastewater treatment as a consequence of increased solubility of the waste activated sludge, enhanced
1132 release of biodegradable organics and favoured growth of hydrolytic microbes and short-chain fatty
1133 acid producers over present methanogens (Huang et al., 2015; Xu et al., 2019). A lot of information is
1134 available about metal (Cd, Pb, Cu, Fe, Zn, Mn, Ni, Hg and Al) remediation for contaminated soils and
1135 secondary mining streams such as tailings using biosurfactants such as rhamnolipids and surfactin
1136 (Mulligan, 2017). Unfortunately, information on SLs is rather limited and often dates back two
1137 decades (Mulligan et al., 2001), though recent years interest seems to be restored, *e.g.* the remediation
1138 of contaminated soils (Basak and Das 2014; Arab and Mulligan 2016; Yang *et al.* 2016; Qi *et al.*
1139 2018). In the context of efficient resource management, bioleaching of Cu out of secondary fayalite
1140 material using acidic SLs as lixivants was reported by Castelein *et al.* (2021), steered by a Cu(0)
1141 corrosion-based and CuS solubilising mechanism. The use of SLs in metal mining context is also
1142 exploited by Dhar *et al.* (2019) in bioflotation processes, where CuS could be collected by acidic
1143 glycolipids including SLs through a chemisorption binding process between the metal ions and the
1144 carboxylic function. Independently of the affordability of SLs, the biggest concern is the selective
1145 antimicrobial activity of SLs and the negative effect it could have on the synergistic effects of the
1146 contaminant degrading and previously balanced microbiome, resulting in potential growth inhibition
1147 of certain species and the loss of degradation capacity for complex hydrocarbons (Patel et al., 2019).

1148 5.8 *Pharmaceuticals and medicine*

1149 Although SLs are not yet applied for pharmaceutical/medical purposes, a lot of literature can be found
1150 on the matter, especially related to the bio-active properties of SLs, *i.e.* their anti-microbial properties.
1151 Microbial contamination in wounds, *e.g.* after surgery and in burn wounds, and the formation of
1152 biofilms (of pathogenic microorganisms) on medical devices, such as catheters and medical implants,
1153 are a crucial problem in the medical sector (Khatoon et al., 2018). These are increasingly caused by
1154 multidrug-resistant bacteria proving the urge for alternative treatments (van Duin and Paterson, 2016).
1155 Therefore, as in other fields, the antimicrobial and antifungal activity of different SL forms against
1156 pathogenic species is a much discussed topic. It was recently reviewed in detail by Solaiman et al.
1157 (2018). Not mentioned in this review are the *in vivo* effects of SLs on wound healing, examined using

1158 a cream containing acidic SLs. The wound healing with the SL cream was accelerated in comparison
1159 to the control without affecting the histology of the healing wound (Lydon et al., 2017). SLs also
1160 appear to exhibit antiviral activity in combination with acceptable cytotoxicity levels suggesting their
1161 use as therapeutic agents in preventing or treating viral infections. To date, they have been described
1162 as effective antiviral agents against both the human immunodeficiency virus (HIV) (Shah et al., 2005)
1163 and the herpes virus (Borsanyiova et al., 2016; Gross and Shah, 2007). Shah et al. (2005) also showed
1164 that SLs could be used as effective spermicidal and virucidal agents. In the late 90's it was shown that
1165 SLs could induce cell differentiation instead of cell proliferation in human promyelocytic leukaemia
1166 (HL60) cell lines and could also inhibit protein kinase C activity within the HL60 cells (Isoda et al.,
1167 1997), a characteristic of an effective anti-tumour agent. Anti-cancer activities were demonstrated on
1168 human hepatoma cells of H7402 (Chen et al., 2006), and on human pancreatic (HPAC) and cervical
1169 cancer (HeLa and CaSki) cells (Fu et al., 2008; Li et al., 2017), after which the apoptotic response was
1170 further clarified (Nawale et al., 2017). However, Callaghan et al. (2016) found that lactonic SLs also
1171 reduced viability of normal human colonic and lung cell lines *in vitro* next to colorectal cancer cell
1172 lines. Also, an increased number of intestinal polyps was observed in Apcmin^{+/-} mice after
1173 administration of 50 mg/kg of lactonic SLs for 70 days. Harmful effects are possibly not negligible,
1174 being an important aspect of further investigation. Next to a bio-active function, molecular self-
1175 assembly of SLs provides potential in pharmaceuticals as an encapsulation component of drugs, which
1176 can be used for controlled drug delivery purposes (Baccile et al., 2016b). Although some in depth
1177 research has been executed and some opportunities seem to exist, the application of new therapeutics
1178 in the pharma/medical sector is highly regulated (*i.e.* GMP and high purity/uniformity required) and
1179 associated with a lengthy registration process. This sector is also less prone to sustainability and/or
1180 bio-based claims compared to other sectors applying surfactants. Nevertheless, the formulation of
1181 drugs and especially paediatric drugs might hold some opportunities as also here some 'classic'
1182 pharmaceutical products keep expanding, leading to withdrawal from the market (Meyers et al., 2020;
1183 Siramshetty et al., 2016). Of course, as not-active agent, SLs would have to be included in the relevant
1184 pharmacopoeias for its use in a certain market(s) which should be justified by the commercial use.
1185 Biosynthesis: unravelled and bended towards the biosynthesis of novel biochemicals

1186 **6 Biosynthesis: unravelled and bended towards the biosynthesis** 1187 **of novel biochemicals**

1188 As described above, sophorolipids are produced as a mixture of different congeners which can in
1189 general be divided into different types of acidic, lactonic and bolaform SLs (section 2). Due to the
1190 biological nature of the production processes, these mixtures are prone to batch to batch variation,
1191 which also gives rise to variation in physiochemical and self-assembly properties (section 5.1).
1192 Although the market is looking for an increased variety of biosurfactants, the in-product variety should

1193 be minimised to limit batch to batch variation, *i.e.* the uniformity should be increased. Increasing
1194 product variety and uniformity can be achieved through process and/or strain engineering. Product
1195 variety can also be increased through screening for new natural producers. In this section, advances on
1196 expanding the inter-product variety of sophorolipid/glycolipid types and on decreasing the in-product
1197 variety, are described, with a focus on the most studied SL producing organism, *Starmerella*
1198 *bombicola*.

1199 The full SL biosynthetic pathway has been elucidated for *S. bombicola* (figure 5). It starts with
1200 the terminal or subterminal hydroxylation of a fatty acid (preferably oleic acid) by the CYP52M1
1201 cytochrome P450 mono-oxygenase enzyme (Huang et al., 2014; Van Bogaert et al., 2013). This
1202 hydroxylation step is followed by two glycosylation steps performed by two distinct
1203 glycosyltransferase enzymes both utilising UDP-glucose as a glycosyl donor, finally resulting in the
1204 addition of a sophorose moiety on the hydroxylated lipophilic tail. The first glycosylation is performed
1205 by the UGTA1 glucosyltransferase enzyme (K. M. J. Saerens et al., 2011c), while the second
1206 glycosylation is performed by a second glycosyltransferase enzyme, UGTB1 (K. M. Saerens et al.,
1207 2011b). The resulting acidic or ‘open form’ sophorolipid can undergo acetylation on the sophorose
1208 moiety by an acetyl transferase enzyme, transferring an acetyl group from acetyl-CoA to positions 6’
1209 and/or 6’’ of the sophorose moiety, thus resulting in a mixture of non-, mono- and di-acetylated acidic
1210 SLs (K. M. Saerens et al., 2011b). This biosynthesis of acidic SLs takes place intracellularly and the
1211 acidic SLs are transported out of the cells by a specific SL transporter similar to multi drug transporter
1212 proteins (MDR) (Van Bogaert et al., 2013). A last enzymatic reaction takes place outside the cell, *i.e.*
1213 an intramolecular esterification of the free carboxylic end of the acidic SLs with the 4’’ position of the
1214 hydrophilic sophorose head (Roelants et al., 2016), giving rise to the so-called lactonic or ‘closed
1215 form’ SLs. The enzyme responsible for this reaction, the *S. bombicola* lactone esterase (SBLE), was
1216 identified in the exoproteome of *S. bombicola* is actively secreted (Ciesielska et al., 2016, 2014).

1217 *Figure 5: Left: Depiction of wild type sophorolipid biosynthesis in S; bombicola. A:B: A depicts fatty acyl*
1218 *carbon chain length while B depicts degree of unsaturation, 1: CYP52M1, 2: UGTA1, 3: UGTB1, 4: SBLE, 5:*
1219 *De novo fatty acid synthesis. Upper right: schematic representation of SLs depicted in figure 1 A, B and C.*
1220 *Bottom right: functional group legend.*

1221 The elucidation of the SL biosynthetic pathway, together with the development of molecular
1222 tools for *S. bombicola*, allowed multiple rational engineering strategies to solve the variety and
1223 uniformity hurdles mentioned above. To increase product uniformity, the composition of the wild type
1224 mixture was pushed towards either (100 %) acidic or either (almost 100%) lactonic sophorolipids by
1225 the creation of an *sble* deletion or an *sble* overexpression strain, respectively (Ciesielska et al., 2016;
1226 Roelants et al., 2016). Yet, although uniformity was increased, the abundance of different SL types in
1227 the new mixtures was still quite high, mainly due to the variation in acetylation degrees and the
1228 incorporation of different types of fatty acids into the non-, mono- or di-acetylated products. A knock

1229 out of the acetyltransferase gene gives rise to the production of non-acetylated molecules (K. M.
1230 Saerens et al., 2011a). Relatively simple overexpression and knock out strategies thus showed to
1231 dramatically increase the uniformity of the produced sophorolipids. Increasing the uniformity can also
1232 be achieved by specifically feeding a desired fatty acid or a substrate enriched in the desired fatty acid
1233 (*e.g.* pure oleic acid or an oil enriched in oleic acid, such as high oleic sunflower oil (HOSO)) (Ashby
1234 et al., 2013). The produced SLs will ‘mirror’ the composition of the fed substrate with a preference for
1235 C_{18:1} fatty substrates. Another strategy lies in modifying the substrate specificity of the responsible
1236 biosynthetic enzymes, but this has not yet been described for SL biosynthetic enzymes.

1237 Similarly, several approaches have been applied to increase the product variety, also both
1238 genetic and process engineering approaches. Using genetic engineering to, for example, knock out the
1239 *sble* and *at* genes in *S. bombicola*, allowed the production of a specific and special type of
1240 sophorolipids with interesting properties, *i.e.* bolaform sophorolipids (Van Bogaert et al., 2016).
1241 Bolaform sophorolipids are produced in very low amounts in the wild type SL mixture (Price et al.,
1242 2012), but this modification allowed more uniform and increased production of these compounds, free
1243 from contaminating lactonic SLs. Still, the problem of uniformity remains, as acidic sophorolipids are
1244 co-produced. The ratio of bolaform SL/acidic SL was optimised by applying process development
1245 strategies (non-published results). Knocking out the *fao1* gene in the former strain increased the
1246 stability of the bolaform biosurfactants, since the linking of the second sophorose moiety on the
1247 hydrophobic linker now occurs through a glycosidic bond instead of an ester bond (Roelants et al.,
1248 2018, Van Renterghem et al., 2018). Again, the choice of substrate has an important impact on the
1249 ratio of different molecules formed, as also here ‘substrate mirroring’ applies, grace to the higher
1250 affinity of the CYP52M1 enzyme towards long chain hydrophilic substrates (Huang et al., 2014). In
1251 another example, Takahashi *et. al* (2016) described improved production of alkyl polyglucosides by
1252 deletion of the *fao1* gene, disrupting the long-chain alcohol oxidation pathway. Brakemeier et al.
1253 (1998b) were able to produce such microbial alkyl polyglucosides by feeding 2-dodecanol, showing
1254 again the crucial role of process engineering strategies to increase product variety, besides genetic
1255 engineering (Brakemeier et al., 1998b, 1995; Lang et al., 2000).

1256 *S. bombicola*’s tolerance towards hydrophobic substrates, due to the abundantly present
1257 (alkane inducible) *cyp52* genes, represents also clear opportunities towards the production of different
1258 types of ‘fatty based’ molecules (Geys et al., 2018; Huang et al., 2014; Inge N.A. Van Bogaert et al.,
1259 2009). Proof of concept therefor was given by Roelants et al. (2013), who showed the production of
1260 the bioplastic polyhydroxyalkanoate (PHA) and production of cellobiose lipids. Moreover, Jeziarska et
1261 al. (2019) succeeded in creating a strain capable of producing about 1 g·L⁻¹ free fatty acids (mixture of
1262 C_{18:1}, C_{18:0} and C_{16:0} chain) by knocking out 3 genes: *faa1* (fatty acyl-CoA synthetase), *cyp52m1* and
1263 *mfe2* (multifunctional enzyme type 2), while De Graeve *et. al* (2019) showed that by controlling the

1264 beta- and omega oxidation, by knocking out $\Delta ugtA1/\Delta pox/\Delta fao1$, the production of long chain hydroxy
1265 fatty acids could be obtained up to concentrations of 17.39 g·L⁻¹.

1266 Hitherto, advances in process optimisation are resulting in higher productivities and therefore
1267 lower production costs of SLs (section 4). In this perspective, genetic engineering strategies can be a
1268 valuable ally. The above described strategies to minimise product uniformity, stimulate lower DSP
1269 costs as less impurities have to be separated. Furthermore, strategies to adapt the metabolism of *S.*
1270 *bombicola* for increased SL production have been described. (Takahiro et al. 2017) described a KO
1271 strategy for higher SL production. Moreover, random mutagenesis in combination with high-
1272 throughput screening is described to increase production SL titres (Lin et al. 2019; Zhou et al. 2019;
1273 Ma et al. 2020). Herein, it is of utmost importance that the performance of the selected strains based
1274 on high-throughput screening methods are validated by accurate SL quantification methods.
1275 Furthermore, random mutagenesis can result in production losses by metabolism distortion caused by
1276 off-target effects and therefore thorough strain performance validation is of utmost importance
1277 (Lodens et al. 2019).

1278 The above mentioned rational genetic engineering of *S. bombicola* required the development
1279 of a molecular toolbox, which is, till to date, under construction. The first step taken was the
1280 development of a successful transformation and selection method. Van Bogaert et al (2008,2007)
1281 described the use of a chemical transformation method, where the cells are resuspended in a
1282 transformation mix containing PEG, LiAc, SS-carrier DNA, and (plasmid) DNA. Currently, the use of
1283 an electroporation protocol combined with lithium acetate and dithiothreitol is more common (K. M.
1284 Saerens et al., 2011a). Conventional yeast antibiotics based selection methods were examined but
1285 hitherto only hygromycin and nourseothricin were reported as robust selection markers for *S.*
1286 *bombicola* (Lodens et al., 2017; Van Bogaert et al., 2008). As for auxotrophic markers, *Ura3* selection
1287 (orotidine-5'-phosphate decarboxylase) is the only one described (Lodens et al., 2017; Van Bogaert et
1288 al., 2007). Furthermore, Lodens et al. (2020) focused on the development of a reporter system for *S.*
1289 *bombicola*. Such a reporter system is key in the development of a molecular toolbox since it allows for
1290 screening of promotor and terminator libraries. Characterised parts for enzyme expression in turn
1291 allow for metabolic flux regulation, which aid in solving problems of titres, productivity and
1292 congeners mentioned above. Here the telomere positioning effect involved in sophorolipid synthesis
1293 was shown using the endogenous promoters *pCyp52m1* and *pGapd*. Finally, a huge leap forward in
1294 domesticating *S. bombicola* was also taken by the development of quantitative proteomics,
1295 transcriptomics and metabolomics methodologies, as well as a reverse- transcription quantitative
1296 polymerase chain reaction (RT-qPCR) platform and liquid chromatography- multireaction
1297 monitoring- mass spectrometry (LC-MRM-MS) (Lodens et al., 2019).

1298 Though the first steps for the domestication of the exotic yeast *Starmerella bombicola* have
1299 been taken, there is room for improvement if compared to other yeast species (e.g. *Yarrowia*
1300 *lipolytica*, *Saccharomyces cerevisiae*). For example, improved homologous recombination could solve
1301 the need for 1000 bp long homologous regions. Events in which genomic integration is required could
1302 be improved by altering the non-homologous end-joining pathway and thus increasing the homologous
1303 recombination efficiency. This strategy has already been proven successful in *Yarrowia lipolytica*
1304 (Verbeke et al., 2013), *Rhodospiridium toruloides* (Koh et al., 2014), *Kluyveromyces marxianus*
1305 (Chen et al., 2013) and several other yeasts. By knocking out the KU70 or KU80 proteins in these
1306 yeasts, genomic integration was drastically altered in favour of homologous recombination, and the
1307 need for large homologous regions became redundant. The isolation of ARS/CEN sequences in several
1308 exotic yeast species allowed the use of plasmids and as such the use of more “complex” molecular
1309 tools like Cre-Lox or CRISPR-CAS9 (Wagner and Alper, 2016). The development of CRISPR-
1310 CAS9 can also eliminate the need for selection markers due to the high efficiency of the technique.
1311 This would obviate the need for marker recovery in *S. bombicola*, which is quite laborious and
1312 frequently required, as only three markers are available. CRISPR-CAS9 has been developed in
1313 numerous non-conventional yeasts, e.g. *Yarrowia lipolytica* (Schwartz et al., 2016) and
1314 *Kluyveromyces lactis* (Spohner et al., 2016). Also, if the tailor-made biomolecules are to achieve
1315 industrial relevance, a (much) more extended library of characterised regulatory parts will be required
1316 (both for transcription and translation). Taken together, this will allow more efficient metabolic
1317 engineering and might open the door to further counteract the issue with SLs produced in mixtures.

1318 **7 Industrial perspectives**

1319 The interesting surfactant and antimicrobial properties of sophorolipids in combination with high
1320 natural productivities and sustainable nature (biological process based on biobased feedstock, good
1321 biodegradability) has resulted in their application in a range of commercialised B2C products and
1322 other applications, as discussed in section 5. Although the industrial interest in biobased surfactants is
1323 clearly on the rise, the production volumes of sophorolipids remain modest (two-digit tonne scale in
1324 Europe). This is mainly because of the high costs of sophorolipids (20-30 euro·kg⁻¹ in Europe) in
1325 comparison with classic surfactants (bulk surfactants typically cost around 1 euro/kg, while chemically
1326 produced biobased surfactants, such as APGs, are typically sold at between 3-6 euro·kg⁻¹). The main
1327 two reasons for these high costs of sophorolipids are 1) the low scale combined with the fact that 2)
1328 industrial microbiology for the large-scale production of biochemicals, such as microbial
1329 biosurfactants, is still a relatively ‘young’ technology compared to mature and fully optimised
1330 chemical production technologies. Clearly this is a catch-22 situation, where the high costs of
1331 sophorolipids prevent an increase in the production volumes and vice versa. When the production
1332 scale and volumes would be increased to let’s say the production of a few thousand tonnes of SLs per
1333 year, the prices could drop below 10 euro·kg⁻¹ depending on substrates, set ups, type of SL molecule,

1334 purification method(s) etc. (Roelants et al., 2018). This drop in price would result in an increase of the
1335 demand and subsequent increase of the production volumes and further decrease of the price, as such
1336 creating a clear growth of the market and escaping the catch-22 situation.

1337 However, this required and substantial increase of production volumes requires quite extensive
1338 capital investments. Although the productivities of the SL production process are typically quite high
1339 (an average volumetric productivity of about $2 \text{ g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ can be easily reached), a production volume of
1340 about 10 000 tonnes of SLs per year would still require an estimated (conservative) bioreactor capacity
1341 order of magnitude of 1000 m^3 plus the required associated equipment for the purification/downstream
1342 processing. Such capacity increase would require substantial CAPEX investments, which should be
1343 substantiated and backed up by a clear market pull for these volumes at the projected price and
1344 requires substantial ambition from the industry. This pivoting point and shift in ambitions from the
1345 industry might have been reached, seen Unilever's recent statements towards their ambition to
1346 completely eliminate fossil feedstocks for the production of cleaning products by 2030 and their
1347 budget reservation of 1 billion euro to do so (Macmillan, 2020). Their aim, together with that of
1348 Evonik, to build a multi thousand tonne scale microbial biosurfactant (rhamnolipid) production plant
1349 in Europe (Evonik, 2019; Roelants and Soetaert, 2021) clearly substantiates that ambition. Now that
1350 SLs have been available on the B2B market (commercialised by Evonik) for about 5 years, several
1351 B2C companies have had the chance to test sophorolipids in their applications.

1352 To our knowledge, industrial interest is there, but price is still a crucial issue. Once large scale
1353 procurement can be guaranteed, investments in large scale production capacity should not be too far off.
1354 This could be done by existing or new players in the field as several industrial parties have expressed
1355 their will to invest in this field and some new players such as Locus, Holiferm and Croda (Snaith, UK)
1356 have expressed their ambitions towards large scale SL production. We thus expect the world to look
1357 quite different in five years from now and predict a bright future for microbial biosurfactants. Whether
1358 sophorolipids will take the same flight as rhamnolipids depends on their (combination of)
1359 functionalities and the will of B2C companies to formulate these compounds in their products because,
1360 besides being green, biobased and mild, the products also need to do the job.

1361 **8 Conclusive remarks**

1362 The interest in and impact of sophorolipids is increasing exponentially. Therefore, this review
1363 provides academic and industrial stakeholders the necessary critical assessment to look back at and
1364 extend the sophorolipid tale, from beehive to bioeconomy. Take home messages are:

- 1365 - The genuine SL producing organisms are located in the genera of *Starmerella* and
1366 *Pseudohyphozyma*.

- 1367 - As for the *Starmerella* species in their natural habitat, it has been postulated that SLs are
1368 produced as extracellular storage compound ('personal hoard') in combination with antimicrobial
1369 activity.
- 1370 - Qualitative strain and structure identification are of utmost importance as previous inadequacies
1371 led to questionable reports.
- 1372 - To utilise SLs to their full extend, process optimisation has been performed to increase production
1373 performance. Hitherto, *in-situ* product recovery in combination with high biomass concentrations
1374 showed promising results. For further process optimisation reports, it is advised to use correct and
1375 standardised terminology. Moreover, production processes should be optimised for overall
1376 production cost and environmental impact. An increase in production process performance
1377 alongside an increase in production capacity will decrease the production cost and result in an
1378 increased application potential. In this perspective, the personal care and cleaning sector are
1379 playing a pioneering role as they already have sophorolipids in their formulations. Subsequently,
1380 the economy of scale will make the production price accessible for crop protection and food
1381 applications. If the necessary investments for production scale-up are performed, we expect a
1382 bright future for sophorolipids and other microbial biosurfactants.
- 1383 - The strength of SLs lies in their function as both (bio-)active component and formulating agent.
1384 Additionally, the 'green nature' (high biodegradability, biological production) and mild properties
1385 of sophorolipids increases the demand for SLs.

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Table 1




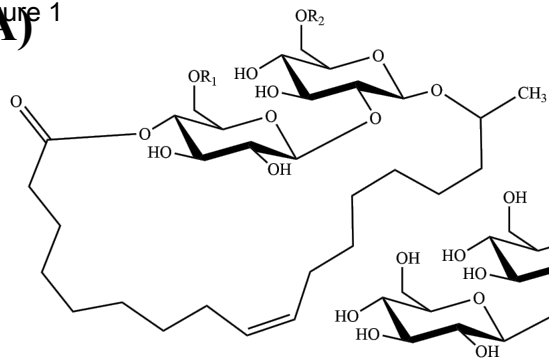
| Yeast species | Fully acknowledged produced SL structures | | | Other | Acknowledged species ID | Acknowledged structure ID | References |
|--|--|---|---|-------|-------------------------|---------------------------|---|
| |  DiAc |  MAc |  NAc | | | | |
| <i>Starmerella riodocensis</i> | ■ | ■ | ■ | | | | (Kurtzman et al., 2010) |
| <i>Starmerella stellata</i> | ■ | ■ | ■ | | | | (Kurtzman et al., 2010) |
| <i>Starmerella kuoi</i> | ■ | ■ | ■ | | | | (Kurtzman, 2012; Kurtzman et al., 2010; Price et al., 2012) |
| <i>Starmerella bombicola</i> | ■ | | | ■ | a | | (Kurtzman et al., 2010; Tulloch et al., 1967; Tulloch and Spencer, 1968) |
| <i>Starmerella apicola</i> | ■ | ■ | ■ | ■ | ■ | ■ | (Kurtzman et al., 2010) |
| <i>Starmerella batistae</i> | ■ | | | ■ | b | | (Konishi et al., 2008) |
| <i>Starmerella floricola</i> | ■ | | | | | | (Imura et al., 2010; Konishi et al., 2017, 2016) |
| <i>Starmerella rugosa</i> | | | | | | X | (Chandran and Das, 2011) |
| <i>Rhodotorula mucilaginosa</i> | | | | | | X | (Chandran and Das, 2011) |
| <i>Pseudohyphozyma bogoriensis</i> | | | | | c | | (Ribeiro et al., 2012a; Shin et al., 2010; Solaiman et al., 2015; Tulloch et al., 1968) |
| <i>Cryptococcus VITGBN2</i> | | | | | | X | (Basak et al., 2014) |
| <i>Cyberlindnera samutprakarnensis</i> | | | | | | X | (Poomtien et al., 2013) |
| <i>Lachancea thermotolerans</i> | | | | | | ± | (Mousavi et al., 2015) |
| <i>Rhodotorula babjevae</i> | | | | | | X | (Cajka et al., 2016; Garay et al., 2017; Sen et al., 2017) |
| <i>Wickerhamiella domercqiae</i> | | | | | | X | (Li et al., 2016) |
| <i>Candida tropicalis</i> | | | | | | X | (Chandran and Das, 2012) |
| <i>Wickerhamiella anomalus</i> | | | | | | X | (Souza et al., 2017; Thaniyavarn et al., 2008) |
| <i>Candida albicans</i> | | | | | | X | (Yang et al., 2012) |

Table 2

| Sopporolipid type | Micellar (L ₁) | | Vesicle | | Lamellar | Fibre | Columnnar | Minor phases | | Reference |
|---|--|--------------------------------|------------------------------------|---------|----------|---------------------------|---|--------------------------|-------------|--|
| | sphere | cylinder | SUV | MLV | | | | Platelets | Ill-defined | |
| <i>N</i> Ac acidic (<i>C18:1-cis</i>) | 4 < pH < 10, C < 5 wt% | pH 4.5, 5 < C (wt%) < 20 | | | | Mix with N° 13 pH < ~7 | | pH > 8 | | (Baccile et al., 2016b, 2011; Cuvier et al., 2015b; Dhasaiyan et al., 2017; Penfold et al., 2011; Zhou et al., 2004) |
| Acetylated acidic (<i>C18:1-cis</i>) | 2 < pH < 7 | | | | | 3 < pH < 7.5 ## | | pH < ~7 | | (Baccile et al., 2017a; Penfold et al., 2011) |
| Acidic (<i>C18:1-trans</i>) | pH > 7.5 | | | | | pH > 8 | | | | (Dhasaiyan et al., 2018, 2013) |
| Acidic (<i>C18:0</i>) | pH > 7.5 | | | | | 3 < pH < 7.5 | | pH > 8 | | (Baccile et al., 2016b; Cuvier et al., 2014, 2015b; Dhasaiyan et al., 2018, 2013) |
| Acidic (<i>C16:0</i>) | pH > 7.5 | | | | | 7.5 < pH < 3 | | | | (Baccile et al., 2021a) |
| Acidic (<i>C18:3-cis</i>) | | | Neutral pH | | | | | | | (Dhasaiyan et al., 2014) |
| Acidic (<i>C22:0₁₃</i>) | pH > 7.5 | | | pH < ~7 | pH < 4 | | | pH > ~7 | | (Baccile et al., 2017b) |
| Lactonic (<i>C18:1-cis</i>) | | | Neutral pH 1 < C (mM) < 5 | | | | | Neutral pH (C > 7 mM) | | (Penfold et al., 2011) |
| <i>B</i> olaform sophorosides (<i>C18:1-cis</i>) | C < 10 wt% | | | | | | | | | (Van Renterghem et al., 2018) |
| <i>B</i> olaform sophorosides (<i>C16:0</i>) | T > 28°C | | | | | T < 28°C | | | | (Baccile et al., 2018) |
| Aminyl (-NH ₂) (<i>C18:0</i>) | pH < 7.5 | | | | | 7.5 < pH < 10 | | pH < 7.5 | | (Ba et al., 2020) |
| Alkynyl (-C≡CH) (<i>C18:1-cis</i>) | T > ~52°C | | | | | T < ~52°C | | | | (Ba et al., 2020) |
| Peracetylated porphyrin sopporolipids | (R ¹ =R ² =OAc) T < T _e (~36°C) | | | | | | (R ¹ = OAc, R ² =OH) (R ¹ =R ² =OH) T < T _e (~36°C) | | | (Mekala et al., 2018; Peters et al., 2020, 2019) |
| Other SLs [#] | C < 10 wt% | | | | | | | | | (N. Baccile et al., 2019) |

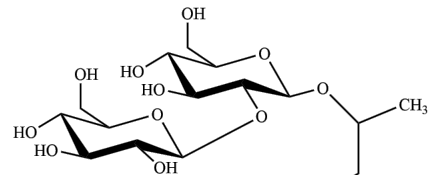
Figure 1

A)

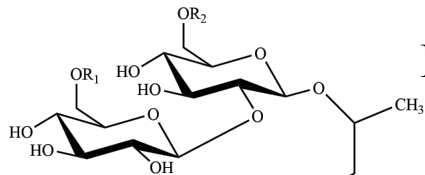


B)

[Click here to access/download;Figure;Figure 1.pdf](#)



C)



D)

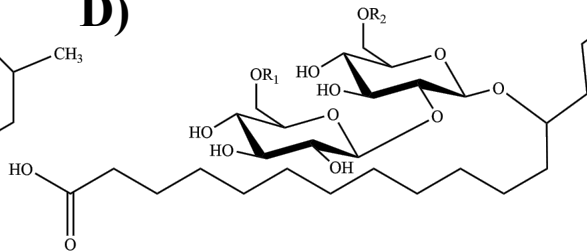
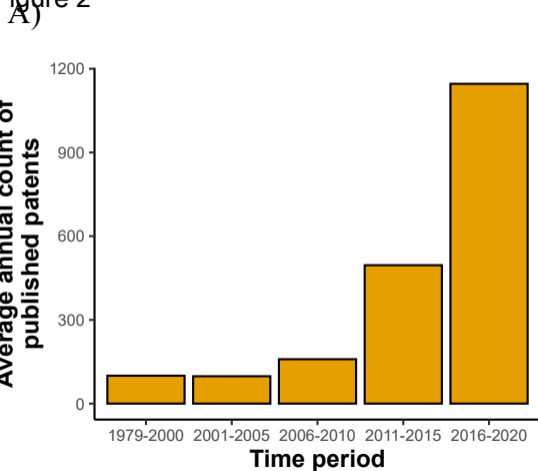
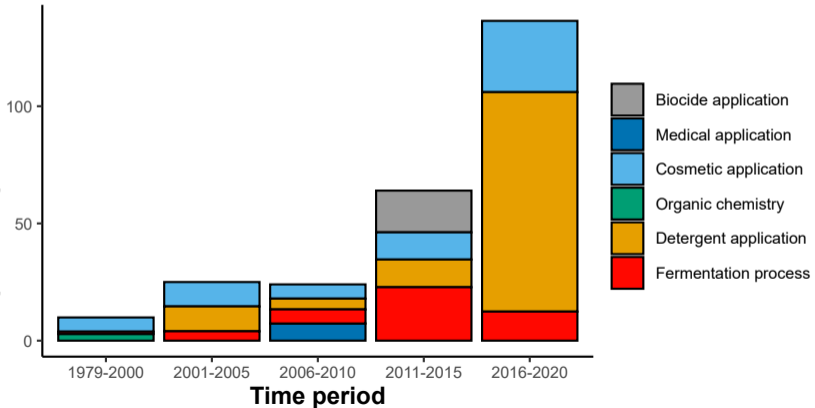


Figure 2



B)

Average annual count of top 10 IPC patent codes



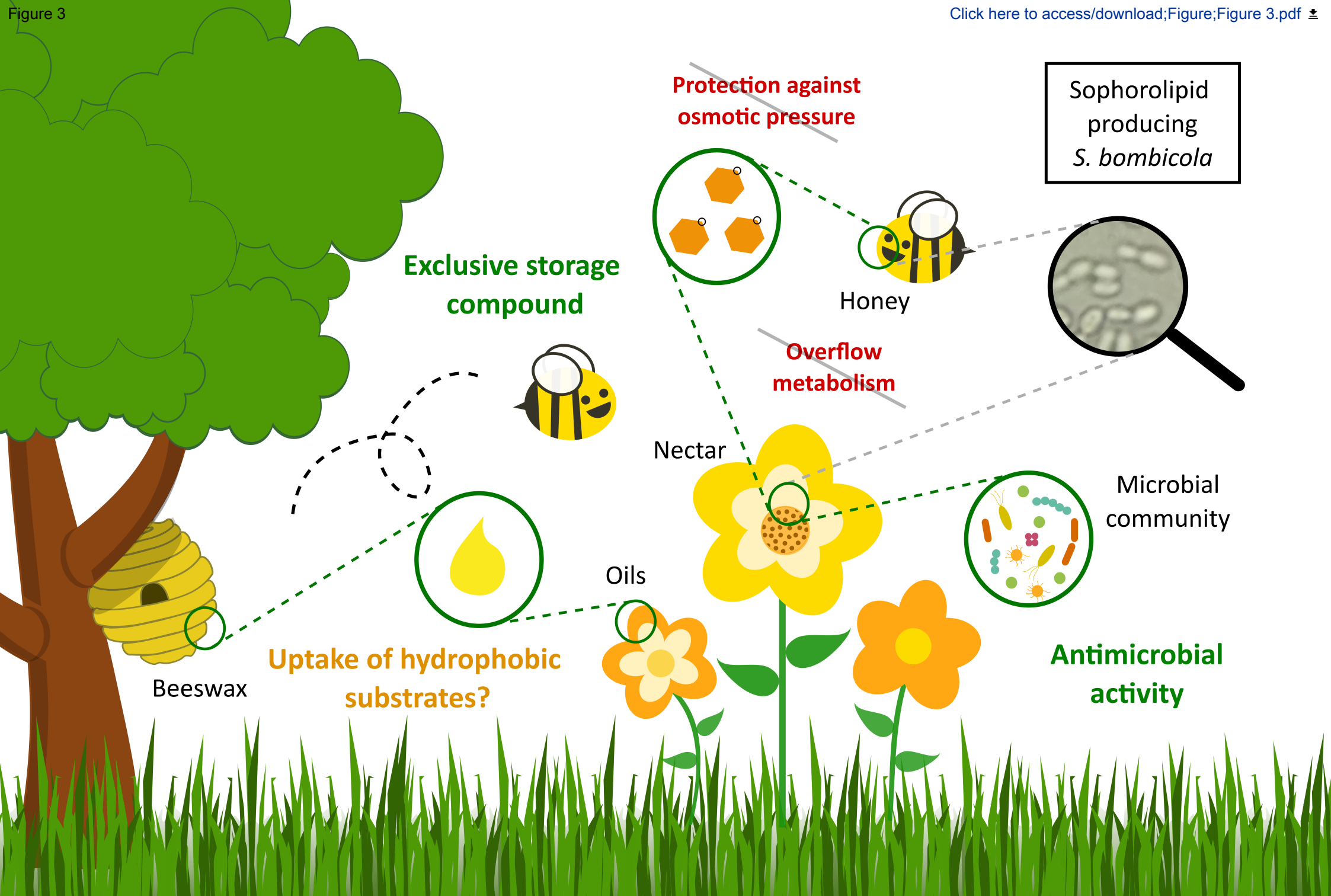
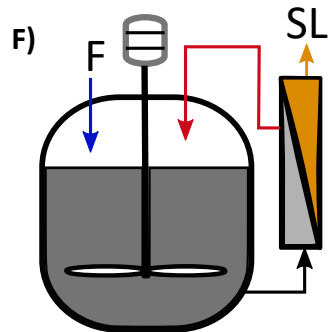
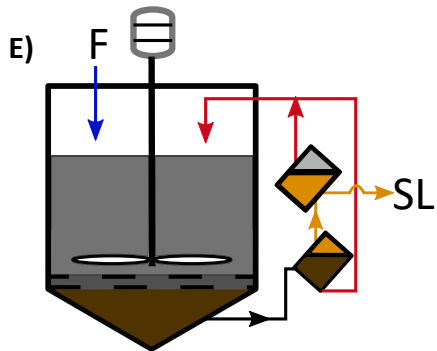
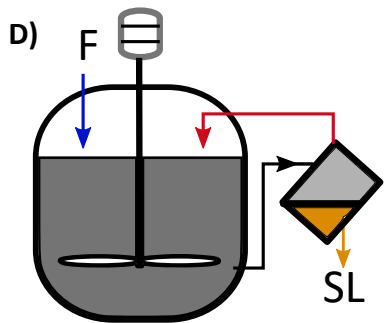
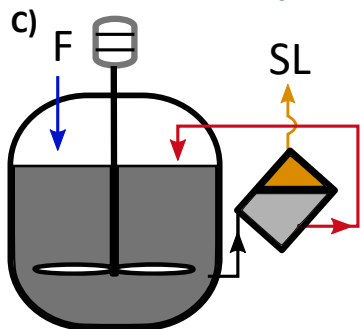
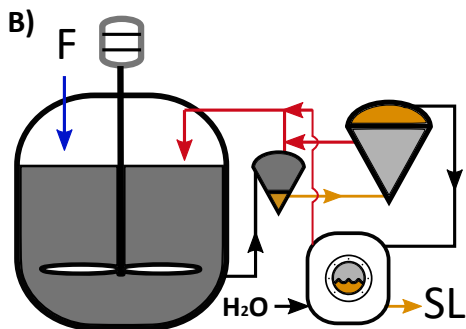
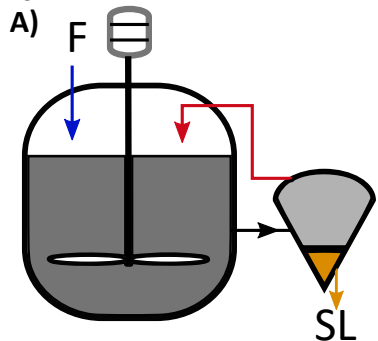


Figure 4

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- I) 
- II) 
- III) 
- IV) 

Figure 5

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