Development and Production of Tailor-Made Biosurfactants.

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Abstract

Two main factors that have limited commercialization of (microbial) biosurfactants are firstly the limited structural variety, secondly the biological generation of mixtures of molecules in combination with batch to batch variation and thirdly the high production price due to low inherent productivities, small scale and/or a lack of process knowledge/optimization.

A solution can be offered by applying an integrated bioprocess design (IBD) approach to increase uniformity and variety and decrease production costs. Strain generation through genetic engineering is followed by thorough production process development (fermentation and purification), with feedback coupling to the strain level. Subsequent scale up on one hand enables assessing the scalability of the processes and performance of techno-economical and LCA analyses, but on the other hand also results in the generation of kg (new) biosurfactants. This again enables dedicated application research in a variety of applications. This approach will be explained for one of the showcases of biosurfactant production: the yeast *Starmerella bombicola*, more specifically a new strain producing the new and innovative bola sophorolipids (bola SLs).

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Introduction

The global surfactant market, worth about 36 billion dollars[1] is characterized by hundreds of different structural variants, which are found in a wealth of applications, from construction and food to precision cleaning industries. About half of this huge production volume is used in household and laundry detergents, while the other half is employed in various industries; e.g. (oilfield) chemicals, mining, paints and coatings, textile and paper, agrochemicals, industrial emulsions, construction, food processing, pharmaceuticals, cosmetics etc. [2]. An emerging class of surfactants are the so called biosurfactants. Biosurfactants or 100 % biobased surfactants can be produced chemically or biologically, starting from natural and renewable building blocks, such as sugars and plant oils as substrates, offering a renewable and 100% biobased alternative to the traditional (petrochemically produced) surfactants. Bio based surfactants constitute about 3 % of the global surfactant market [3], a volume which is mainly dominated by chemically produced biosurfactants like MESs, APGs and sugar esters. The biologically produced biosurfactants can be obtained through extraction from plants (e.g. cardolite from cashew nut shells), biocatalysis (e.g. enzymatic sugar esters) and fermentation (e.g. rhamnolipids, sophorolipids, surfactin and xylolipids) better known as microbial biosurfactants. In the latter, biological production process (fermentation), natural building blocks, such as sugars and plant oils or even waste/side streams, are employed. The ecological advantage associated with such processes, together with the rising awareness towards sustainability, clearly underpins the market potential of biosurfactants also translated in the patenting activity [4]. In this book, the last class of biosurfactants, i.e. microbial biosurfactants has been described in detail. Although a lot of research has been devoted to this class of biochemicals, a very limited amount is currently available on the market, estimated by the authors to account for only a few thousand metric tons/year and thus below 0.1 % of the global surfactant market. This limited market penetration is caused by three main reasons: First of all, microbial biosurfactants are generally produced as complex mixtures e.g. rhamnolipids can be found as mono- and dirhamnolipids and the chain length of the hydrophobic monomers can vary [5] sophorolipids can be found in acidic and lactonic forms and chain lengths, site of hydroxylation, saturations degree etc. [6,7], lipopeptides vary in the constituting amino acids and fatty acids [8,9], while MELs vary in their acetylation and acylation degree [10]. This situation is schematically summarized in Fig. 1, clearly for all the biosurfactants, mixtures are produced, and when one speaks about e.g. “sophorolipids”, one is speaking about a mixture of between 20 and 100 compounds.

Fig. 1: Schematic representation of the structural variety of biosurfactants (1) Rhamnolipids, (2) Mannosylerythritollipids and (3) Sophorolipids. Top: the classic representation of the biosurfactants, Below: a schematic representation of the diversity of molecular structures captured within the biosurfactant types.

The fact that mixtures are produced as such is not the biggest issue though. If a mixture does the job, the industry and end users will be satisfied, irrespective of it being a mixture. However, the fact that the ratio of the respective compounds is prone to variation (culture/growth conditions, substrate variation, medium components etc.) [11–16], in combination with the fact that the different congeners are often associated with very different properties (e.g. lactonic versus acidic sophorolipids [17], MELs with a variation of the ac(et)ylation degree [13,18], RLs with one or two rhamnose moieties [19], etc. can result in highly confusing and undesired situations. One batch of a specific product can perfectly show completely different functionality as compared to another batch of supposedly the same product. The occurrence of such issues is completely unacceptable from a market perspective, where products must comply with the specifications defined by the manufacturer[20].

A second reason for the small market share, is the fact that the molecular variants of microbial biosurfactants, produced at acceptable efficiencies (and thus acceptable production costs) by the respective microorganisms, is currently too low as compared with synthetic alternatives. In formulation business, mixing and combining ingredients to get to a certain functionality, requires the availability of choice. Indeed, a range/a portfolio of hundreds of synthetic products is available on the market, while only a handful of microbial biosurfactants is available. Last, but not least, price is a major issue. This last issue is a constraint for most new biotech

products and processes, according to several biotech startups (personal communications). This is mostly due to the fact that such new products cannot profit from the economy of scale yet, while the associated production processes (strains, fermentation and purification) have not yet been thoroughly optimized. Moreover, petrochemical production plants are mostly fully depreciated. To summarize the above in three words: uniformity, diversity and efficiency are key issues to resolve for increasing the market share of microbial biosurfactants.

In this paper, the approach followed by researchers at InBio.be and BBEPP to resolve these issues and as such bring (new-to-nature) glycolipid biosurfactants to the market, is elaborated. In this so-called 'integrated bioprocess design' (IBD) approach; strain engineering, process development and scale up and dedicated application research are closely interconnected. Iteration between the different 'unit operations' enables early identification of bottlenecks and the definition of solutions along the way. This approach tackles the three bottlenecks mentioned above: uniformity, diversity and efficiency.

Increasing molecular variety and uniformity through strain engineering

The working horse at the core of this 'IBD' strategy is the 'exotic' yeast *Starmerella bombicola* [21]. 'Exotic', because it is not a well-described lab strain for which molecular tools are readily available, like e.g. *Saccharomyces cerevisiae*. Molecular tools are required for genetic engineering to be possible. This first requirement/bottleneck was defined by the research group of Professor Soetaert fifteen years ago as the way forward to increase molecular variety and strain efficiency (decreasing production costs), while reducing product complexity (increase uniformity). His research group, InBio.be, thus set out on the quest to develop such molecular tools for *S. bombicola*, which has resulted in the slow, though eventually exponential expansion of the possibilities. A 'hands on' overview of this endeavor has been recently compiled [22]. The choice for this particular yeast strain to be transformed into a platform organism for (new-to-nature biosurfactants), is the fact that *S. bombicola* naturally produces high amounts of the biosurfactant sophorolipids (SLs) (> 200 g/L; 4 g/L.h) [23]. The biosynthetic pathway of SLs has been elucidated by our lab (ref) and the contributing enzymes described and characterized [24–29].

SLs are a well-known example of glycolipid microbial biosurfactants and are composed of the rare disaccharide sophorose, attached to a (hydroxylated) fatty acid chain, and occur in an 'open' or acidic conformation or 'closed' or lactonic conformation (see Fig. 1). *S. bombicola*, synthesizes these molecules in high amounts from renewable resources and even waste streams [15,30–34], which results in substantial industrial interest. Commercialization of SLs has thus been pursued by several companies, amongst others by Evonik, Soliance and Wheatoleo and application by Henkel, Ecover, Saraya, and Wheatoleo [20]. SLs are thus one of the microbial biosurfactant success stories, being one of the few types that have made it to the market. However, some issues are associated with the natural occurring 'sophorolipids'. As mentioned above, they occur as a mixture of lactonic and acidic congeners (see Fig. 1), which have very different properties. The ratio of lactonic over acidic, varies between the two extremes and is influenced by media and culture conditions [17]. Moreover, variation in the acetylation degree of the hydrophilic head group [24] in the saturation/length of the hydrophobic tail and in its site of hydroxylation (terminal or subterminal, and thus linkage to the sophorose head group) occurs.

Although the biosynthetic enzymes have a certain preference, they are quite promiscuous, giving rise to about 20 'major', and no less than over 100 'minor' homologs in the SL mixture. Several strategies have been described to at least control the lactonic/acidic ratio, as this variation is responsible for the largest part of the functionality shift and in the opinion of the authors, explanatory of the varying statements in the literature concerning SLs physicochemical and biological characteristics. To give an example, lactonic SLs have clear antimicrobial and -viral properties and low foaming potential, while acidic SLs show very low or no antimicrobial activity and foam considerably well. Fermentation and purification based strategies can quite efficiently generate two types of quite uniform SL types i.e. 1. > 95 % diacetylated lacton SLs and 2. 100 % non-acetylated acidic SLs respectively [17,35,36] (see Figs. 1 c and 3) as two structural variants. The last are deduced from the first by applying alkaline hydrolysis [37], as such hydrolyzing all ester functions (acetyl- and lacton functionalities). Several companies also generate/produce a lactonic

SL product, which is partly hydrolyzed i.e. generation of a mixture of acidic and lactonic SLs in a controlled way. This is done for two reasons: increasing the water solubility of lactonic SLs and the stimulation of synergistic effects between the two forms.

Although the industry has thus eventually managed to valorize the (complicated) potential of wild type SLs, genetic engineering offers a more elegant and absolute solution to the abovementioned issues. Indeed, the development of a molecular toolkit enabled us (and some other research groups) to generate a range of new strains producing (new-to-nature) glycolipids (Saerens et al. 2011, 2011b; Roelants et al. 2016; Takahashi et al. 2016; Van Bogaert et al. 2016; Van Renterghem et al. 2017). An overview of the most important molecular structures, which can be produced at productivities similar as the wild type strain is shown in Fig. 2.

Fig. 2: Overview of the glycolipid portfolio based on engineered *S. bombicola* strains for the production of new to nature glycolipids at acceptable productivities developed at the University of Ghent (InBio.be). Acetylgroups (in blue) can be present (varying degrees) or absent.

In this figure it thus is obvious that we have unchained *S. bombicola* as a platform organism for new types of glycolipid biosurfactants. The availability of a battery of very similar glycolipid biosurfactants does not only tackle the 'diversity' criterion and proof of concept for other microbial biosurfactants. Moreover, this is the first time to our knowledge, that there is a realistic possibility to determine in depth structure-function relationships, i.e. investigate the influence of the polar head group (one versus two sugars), the influence of acetylation (no, low, medium or high acetylation degree), the influence of the functionalization of the lipophilic part, etc. on the molecular functionality. Besides these achievements on the first abovementioned key point 'diversity, the second bottleneck, 'uniformity', was also tackled by our research group. For example: a strain was generated which exclusively produces acidic SLs [29] [17] and another one which exclusively produces lactonic sophorolipids, conditions [17].

To come back to the abovementioned integrated bioprocess design (IBD) strategy, one of the new strains/molecules depicted in Fig. 2, i.e. a new type of SLs, so-called non-symmetrical (ns) bolaform SLs, will be used as an example to guide the reader through the several steps of this IBD approach. These ns bolaform SLs were quite recently discovered in the wild type SL mixture in minute amounts [7]. The analytical strength in the early years of SL characterization was probably too low to detect these compounds, resulting in their late discovery compared to 'sophorolipids' in general [41,42]. In contrast to the structure of classic SLs (i.e. acidic and lactonic SLs) (see Figs. 1 c and 3), bolaform SLs consist of two sophorose moieties located on each side of the lipophilic chain. Shortly after the discovery of [7], our lab succeeded in generating a strain that almost exclusively produces these ns bolaform SLs [39]. The term 'non-symmetrical' was later introduced, because the two sophorose head groups are attached to the lipophilic linker through a glycosidic and ester linkage respectively (see Fig. 2). Due to their unique structure, bolaform amphiphiles are promising for a range of applications. Synthetic bolaforms are for example applied for nanomaterial synthesis of the anti-HIV drug Zidovudine® [43]. Bolaform SLs could represent an interesting biological alternative and/or addition. Besides such rather high-end applications, the use of the bolaform SLs in detergent applications is another possibility as this is the market were the major part of industrially produced 'classic' SLs find application. After pursuing the IBD approach to develop and optimize production methods for these new and intriguing molecules (which will be further explained below), it indeed became clear that the molecules have a rather limited stability. This was caused by the non-symmetrical nature of the molecules, as the ester function is prone to (spontaneous) hydrolysis. This instability complicates their production (hydrolysis during fermentation, and more significantly during purification) and would give rise to instable functionality in watery applications. Considering the IBD approach, we thus thought about ways to circumvent this issue (see Fig. 3).

Fig. 3 Schematic representation of the integrated bioprocess design (IBD) approach applied by InBio.be and BBEPP. Strain engineering, process development (fermentation and purification) and dedicated application research are closely interconnected and drive the movement of the new to nature glycolipids along the innovation chain.

A solution was identified by coupling back to the strain/fermentation level, aiming to generate compounds with two glycosidic linkages (i.e 'symmetrical') instead of one glycosidic linkage and one ester linkage (i.e. 'non-symmetrical') as also depicted in Fig. 2 i.e. symmetrical bola sophorosides or disophorosides. A new strain was thus generated which now indeed efficiently produces symmetrical bola sophorosides [40].

As mentioned above, after the development of the non-symmetrical bola SLs producing strain, the IBD approach was followed to enable the final valorization of these molecules. First, the fermentation to increase efficiency/productivity was investigated, which will be described below. In parallel with this, a purification method was developed and thirdly the application potential of these new molecules was investigated. Emphasis on the parameter 'productivity', as the most important parameter influencing efficiency, is used below as this was found to be the most important parameter influencing production cost and described in the third part of this chapter.

Increasing efficiency and uniformity through process development

Both medium composition as feeding regimes and fermentation set up are considered in process development. In terms of medium, both the nitrogen source and the choice of the hydrophobic carbon source were evaluated and the results of the combined optimization efforts are summarized in Table 1. It is clear that all the changes, resulted in a dramatic positive evolution of the ns bola SL productivity (i.e. a 14 fold increase).

Table 1: Summary of the achievements for ns bola SLs productivity along the innovation chain for the process development part of the IBD approach.

	Productivity ns bola SLs (g/L.h)
CSL + Colza oil	0.05
YE + Colza oil	0.19
YE + High oleic sunflower oil (HOSO)	0.22
YE + Oleic acid	0.44
YE + Oleic acid + cell retention	0.63

The last result shown in Table 1 is derived from a continuous fermentation, by applying cell retention in the stationary phase of the fermentation as schematically depicted in Fig. 4a. Seen the high solubility of ns bola SLs (> 500 g/L) and its successful purification using a two-step ultrafiltration process [40], the idea arose to couple fermentation and purification in a full continuous set up (Fig. 4 a and b).

Fig. 4: Schematic representation of a full continuous set up for ns bola SLs production i.e. fermentation and purification (two step filtration). The continuous fermentation with cell retention was successfully performed, but the steady state for constant productivity could only be maintained for 10 days.

Such full continuous set-up would significantly decrease the down-time of the equipment and the coupling of fermentation with purification would be a highly innovative achievement for biosurfactant production. Although full continuous systems can represent some clear advantages, such systems also demand highly robust biosynthesis e.g. constant productivity has to be maintained throughout the process. The productivity as shown in Table 3 (0.63 g/L.h) could be maintained for 10 days, whereas typically only around 0.37 g/L.h was obtained during fed batch fermentations. However, after 10 days of continuous fermentation, the productivity started to drop. It was not entirely clear what was the reason for this drop as most of the constituents of the medium were fed to the yeasts in the influent. We are currently investigating the cause of the productivity drop in detail. Resolving this issue might enable the development of a full continuous system as described elsewhere [44].

Following the IBD approach, in parallel with these fermentation process actions, the strain engineering unit operation was also further considered. These strain engineering strategies were performed at the University (InBio.be) in parallel with the above described process development strategies performed at BBEPP.

Although the abovementioned accomplishments can be considered as breakthroughs in the microbial biosurfactant world, it is clear that to further broaden and optimize the production of new to nature glycolipids, in depth metabolic engineering strategies are required. The latter on the other hand requires the further expansion of the molecular toolkit and an in-depth knowledge of the molecular regulation of SL biosynthesis and its related pathways. The last goals are currently thoroughly being investigated at Inbio.be.

Scale up and techno-economic and environmental profiling

To further investigate the developed production processes on the techno-economic, but also on the environmental level, the developed processes were further scaled up to the 100 L and 15 m³ scale. Such scale up not only enabled us to evaluate the feasibility of the processes, but also resulted in the generation of kg scale amounts of the product (ns bola SLs) for market exploration (see below).

The technical and economic (TE) feasibility of a dedicated non-symmetrical bola sophorolipids (SL) producing plant was subsequently assessed [44]. The impact of the fermentation productivity (0.05 to 4 g/L.h) and the yearly production scale (0 and 10 kTon per year) on the total production cost (CAPEX and OPEX included) is shown in Figure 5.

Fig. 5. Prediction of production costs of ns bola SLs in function of production scale and ns bola SL productivity as generated by an in-house developed model (CAPEX and OPEX included). The 0.05 g/L.h option was not included to allow clear reading of the figure. The plateau for this value was found at around 100 euro/kg.

[45] published a calculated projected production cost of wild type SLs of about 2 euro/kg SLs. However, this group assumed very high production volumes (90 000 ton/year). The absolute minimum level of production cost will always be dictated by the major substrate cost i.e. glucose and oil/fatty acids, together accounting to between 1.5 and 3 euro/kg depending on the used substrates. A clear trade-off between cost (substrate, medium, type of DSP) and efficiency would have to be considered in detail at a certain production scale. Some parameters will be negligible at smaller scale (i.e. CSL versus YE), but might become important at very high scales as such parameters do not scale, like labour for example.

The proposed process and generated data at 15 m³ was also used to calculate the environmental impact of the production and use of ns bola SLs similarly as was performed for acidic SLs [46]. Similarly as described by for acidic SLs by these authors, the environmental impact of ns bola SLs was unexpectedly high as compared to classical surfactants. Biosurfactants are always considered and described as sustainable and green alternatives to classic surfactants. This is a clear illustration of the fact that biobased/green products/processes are not automatically the more sustainable solution. However, the largest part (> 87 %) of the environmental impact was derived from the use of first generation substrates (glucose and oleic acid) as input for the production processes. This impact is derived of the negative influence on the environment of the agricultural processes associated with the production of these substrates.

The use of second generation (2G) substrates or even better, substrates requiring very little fertilization/watering (as the 2G substrates are still indirectly associated with these practices through the generation of the 1G substrates), would supposedly result in a positive effect on the environmental impact if the process efficiencies can remain largely unaffected. The latter was thus evaluated at BBEPP using 2G sugars derived from the bio-refinery company CIMV in combination with a microbial oil.The results will be described in detail elsewhere, but summarizing: this entire (hydrophobic and hydrophilic carbon sources) 2G fermentation for SL production was successful and gave rise to good efficiencies and product of excellent quality. Again, further optimization of the processes would be required to valorize these results. However, seen the outcomes described above, these results were considered as a very positive and innovative result as there have not been a lot reports about/of full 2G processes for biosurfactant production.

Application potential and exploratory marketing

Depending on the application, the market demands cheap and characterized products offering a versatile application range and compatibility with other formulation ingredients. A multitude of companies are active in research on/about production and/or application of SLs [20]. For the ns bola SLs we initially only evaluated their use in ecological detergents as a 'usual suspect' application. Although quite good results were obtained for such applications [40], the current production costs are too high for these types of applications to be a realistic option. When looking at Fig. 5, the production cost (not commercial selling price) of ns bola SLs could reach 30 euro/kg active matter at the current productivity (~0.7 g/L.h) once about 430 tons of this product would be produced/commercialized. We expect that this price should go down for B2C companies to 'massively' apply these compounds in their products. However, as the new molecules have an interesting and innovative structure, they might have properties/application potential for more high-end applications (e.g. pharma, cosmetics, nanotechnology). However, as the possible application potential of the ns bola SLs and with expansion the entire portfolio shown in Fig. 2, is very broad, we are currently applying an exploratory strategy considering a very broad variety of applications/sectors as shown in Fig. 6.

Fig 6. Representation of the potential application markets for the glycolipid portfolio shown in Fig. 3. All of the mentioned markets/applications fields are currently under evaluation in partnerships with academia and/or industry.

The identification of high end applications benefiting from ns bola SLs, would enable their market introduction at higher prices, after which the economy of scale will kick in as shown in Fig. 5. This third part of the IBD approach is also clearly integrated with the other unit operations. For the ns bola SLs for example it became clear during scale up and also during application experiments for wash up liquids, that the molecules were not that stable. This subsequently gave rise to the development of the strain generating the stable symmetrical bola sophorosides [40].

After a suitable application is found, the regulatory aspects still have to be taken into account, which will be different depending on the targeted application (e.g. food versus detergents). Taking this hurdle will be one of the last ones to take before one of the products can make it to the market. This regulatory tract is both very laborious and expensive.

Final thoughts

The abovementioned accomplishments clearly show that applying an IBD approach can enable the valorization of new to nature glycolipid biosurfactants. The unique combination of genetic/metabolic engineering strategies, process development, scale-up and application development is key to valorize the abovementioned technology platform.

Although the abovementioned accomplishments can thus be considered as very valuable proof of concepts in the microbial biosurfactant world, it is clear that there is still room for a lot of improvement. First of all, more in-depth metabolic engineering strategies are required to steer the engineering efforts in a rationalized fashion. The latter on the other hand requires the further expansion of the molecular toolkit, in-depth knowledge of the molecular regulation of glycolipid biosynthesis and its related pathways. The last goals are currently thoroughly being investigated at Inbio.be.

The medium composition is another uncultivated source of improvement. Although we have already performed some improvements as described in this chapter, we have not thoroughly dissected the medium optimization option yet. This is another point where we are currently extensively investing efforts by applying DOE and HTP set-ups. All the above-mentioned efforts were performed aiming to reduce the production costs. However, anno 2017 a balance with the environmental profile of the molecules should also be considered. In this research it was shown that the environmental impact can be drastically decreased by the application of 2G (like) substrates. However, these technologies should first be further optimized, to reach similar efficiencies and cost as 1G substrates. A last and very important factor is the further and expanded exploratory marketing research. First, the determination of basic parameters used to score surfactants/glycolipids in a multitude of sectors/applications will aid the substantiated choice for

more in depth, specific application research.
To conclude we confirm that applying an integrated bioprocess design strategy (IBPD), i.e. considering the entire innovation chain, from genetic engineering through fermentation and downstream processing to final application testing, is key to develop new strains and processes for the industrial production and commercialization of new biosurfactants and with expansion other types of (non-drop in) biochemicals.

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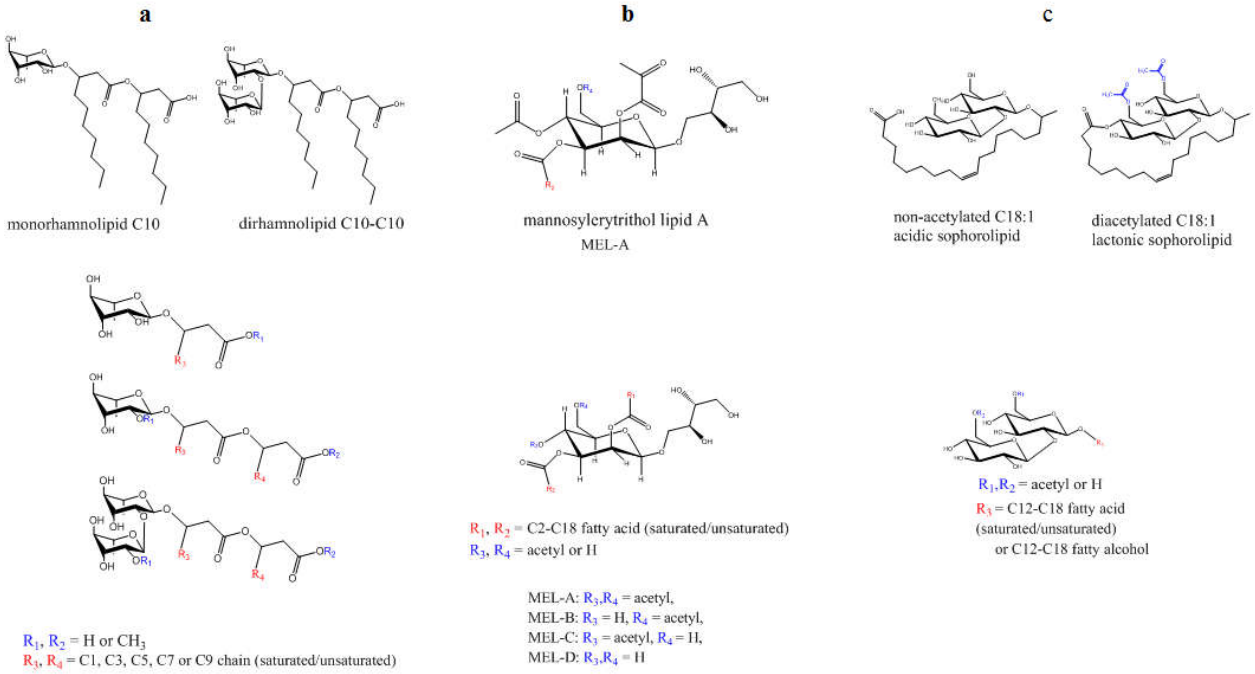

Figure 1

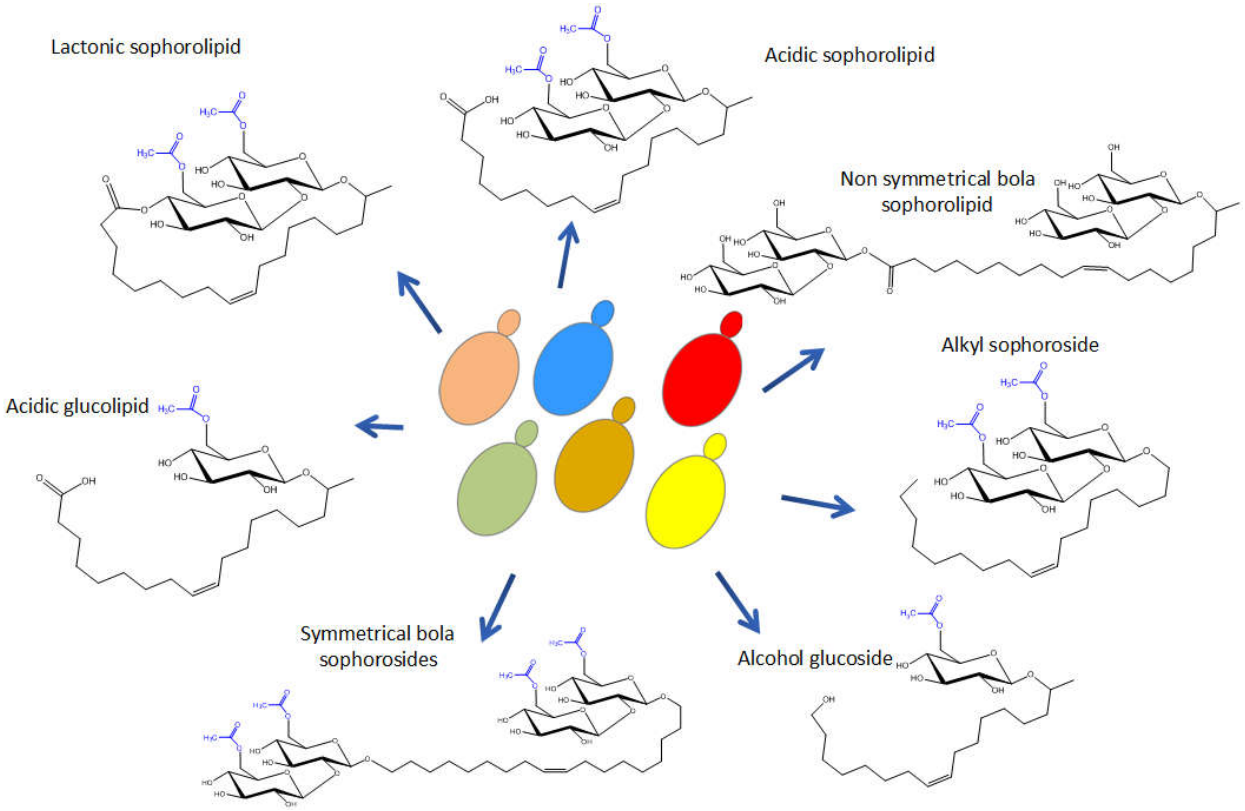

Figure 2

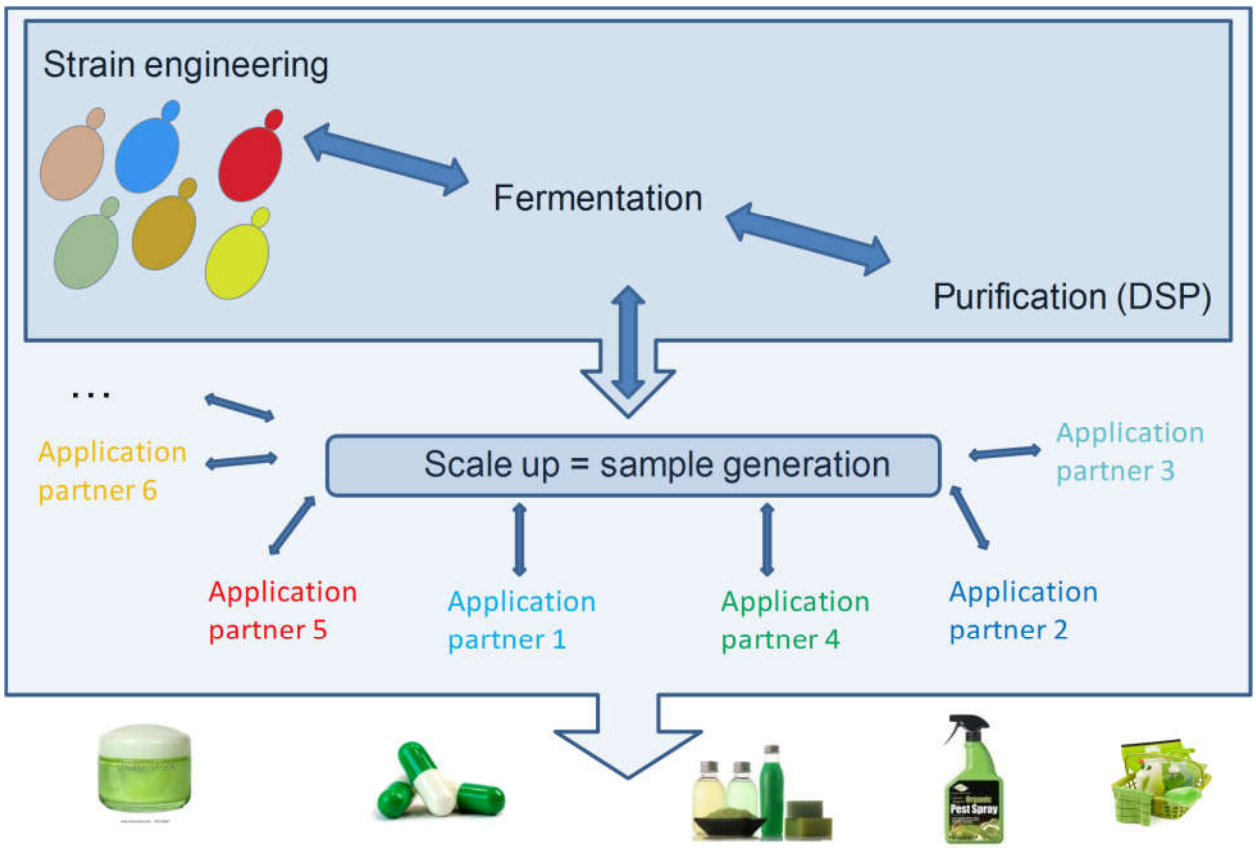

Figure 3

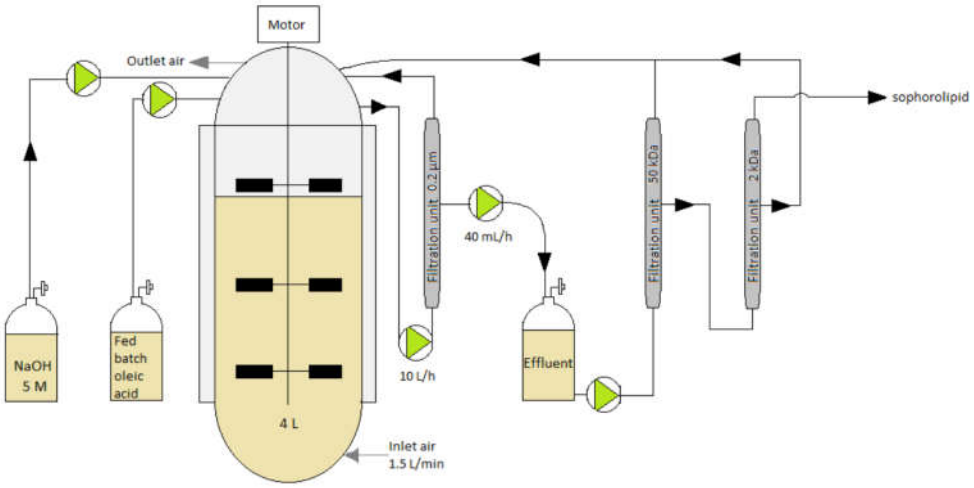

Figure 4

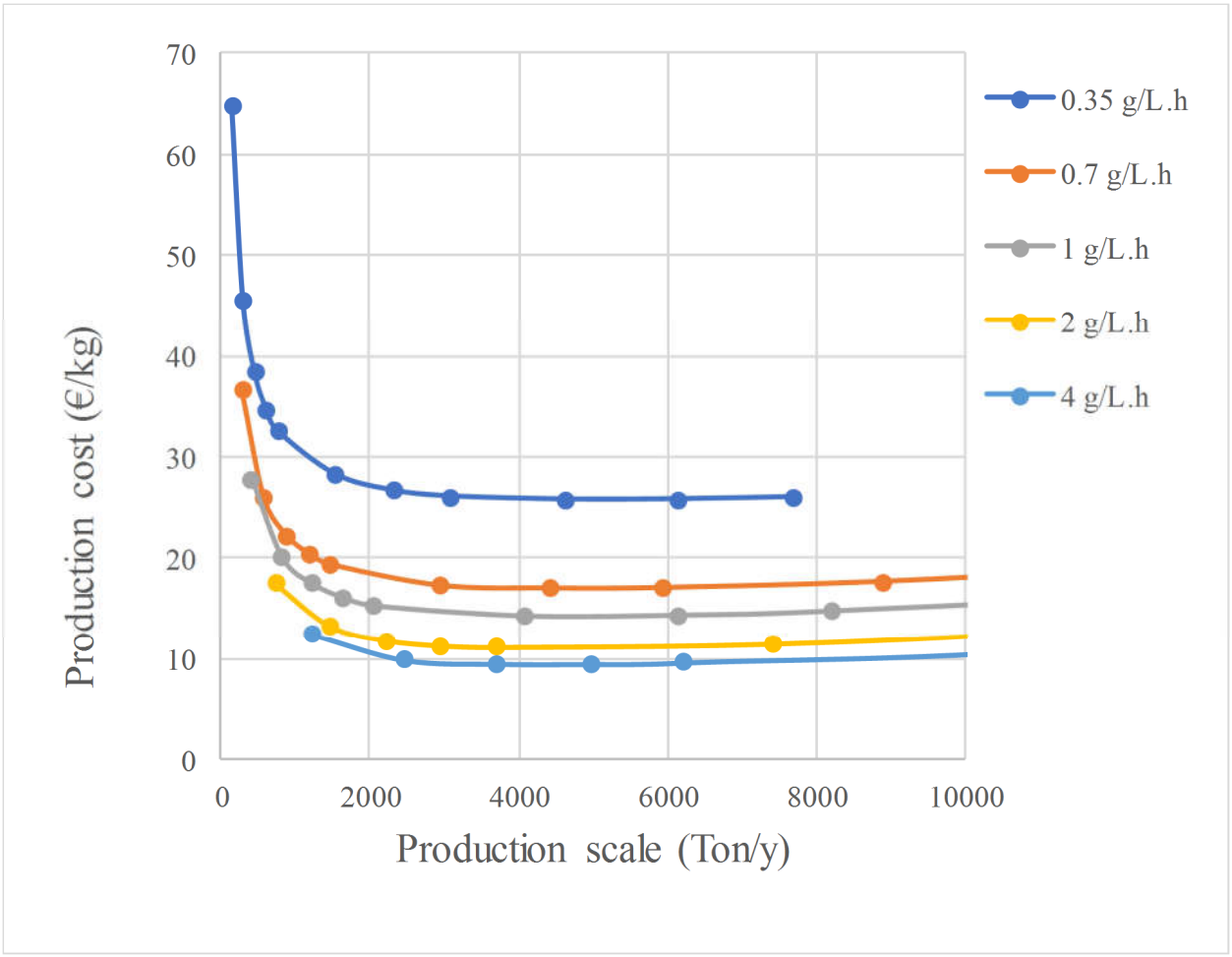
70
60
50
40
30
20
10
0
0
2000
4000
6000
8000
10000
Production cost (€/kg)
Production scale (Ton/y)
0.35 g/L.h
0.7 g/L.h
1 g/L.h
2 g/L.h
4 g/L.h

Figure 5

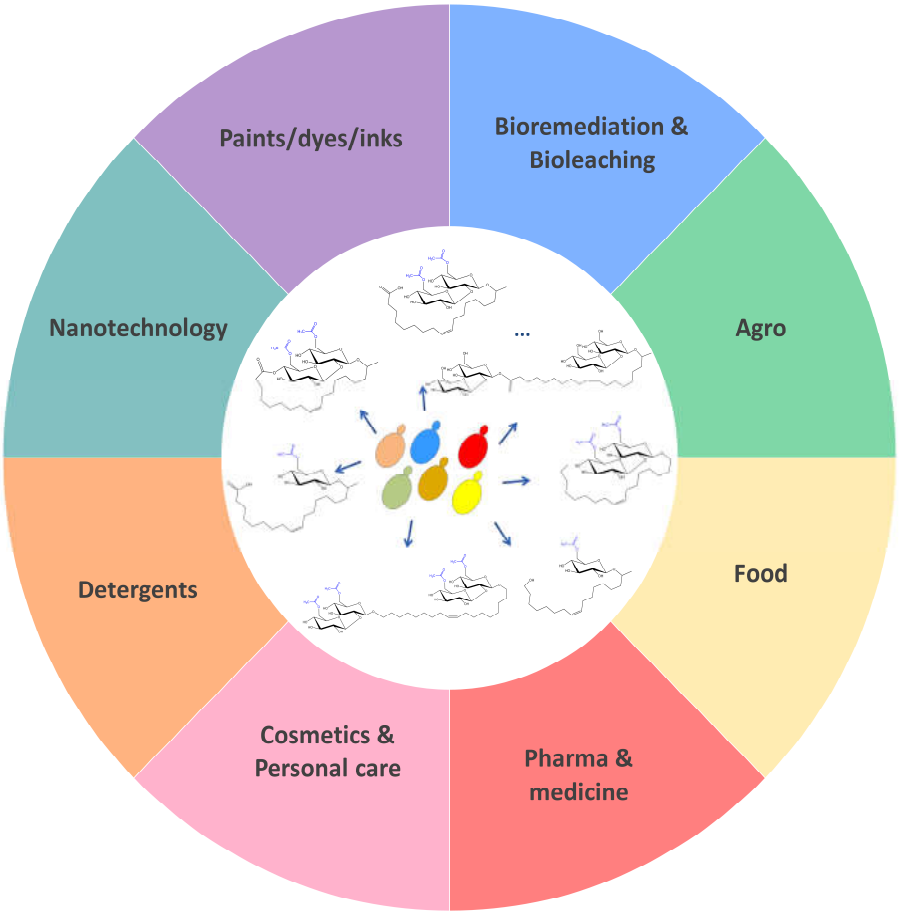
Paints/dyes/inks
Bioremediation & Bioleaching
Agro
Food
Pharma & medicine
Cosmetics & Personal care
Detergents
Nanotechnology
...

Figure 6