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Biosurfactants for the Biobased Economy

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Aims and Scope

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Rudolf Hausmann • Marius Henkel
Editors

Biosurfactants for the Biobased Economy

With contributions by

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Preface

Biosurfactants represent an interesting group of substances in industrial microbiology, which is increasingly gaining research interest. They do not represent a uniform class, but are characterized by their great structural diversity. Structural classes relevant to a future bioeconomy are glycolipids and lipopeptides, which receive special attention in this volume. As such, this volume focuses on these microbially and enzymatically produced biosurfactants of industrial biotechnology.

One reason for the increasing research interest in biosurfactants is the undeniable warming of the earth's climate and the finite nature of fossil resources. In general, this has led to increased use of renewable raw materials as carbon sources in the chemical industry. In this context, the slow but steady paradigm shift in recent years has brought to light the need to replace the substrates previously used for biosurfactant production, especially glucose and vegetable oils that compete with food, with new bioeconomic substrates, especially wood sugars.

In addition, consumer demand for sustainable and carbon-neutral products is also driving the need to replace petrochemically produced chemicals with biochemically produced alternatives based on renewable resources. Already today, some microbial surfactants, namely the glycolipids, mannosylerythritol lipids, rhamnolipids, sophorose lipids, and the lipopeptide surfactin are commercially available on an industrial scale. It is foreseeable that screening and synthetic biology will lead research into new commercially relevant biosurfactants, which will include designer biosurfactants.

It is anticipated that bioproduction of microbial surfactants will bring increased efficiency in the future through metabolic engineering and new bioprocesses to be developed for in situ product separation, ensuring economic competitiveness in an increasing number of applications. Biosurfactants serve these requirements in their entirety. Surfactants, in general, are among the highest volume chemicals and to date have been produced by petrochemical or oleochemical routes. In both cases, environmental and climate issues are evident due to the use of fossil carbon or tropical palm oil, respectively.

Biosurfactants, however, offer additional advantages besides surface activity. As a rule, biosurfactants exhibit specific bioactivities against fungi and other agricultural pests. Here, it is important to further explore and implement this potential as biopesticides.

In this context, it is of particular importance to scientifically prove the presumed ecological benefits of biosurfactants and make them transparent through well-established life cycle assessments. So far, however, there is a need for research in the creation of the biotechnological and process engineering data basis.

With the volume presented here, the world's leading experts in the various fields of biosurfactant research present their views on the subject. We are very pleased to present deep insights into the current and future development of biosurfactant research with this collection of articles.

Stuttgart, Germany

Rudolf Hausmann
Marius Henkel

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Industrial Perspectives for (Microbial) Biosurfactants

Sophie L. K. W. Roelants and Wim Soetaert

Contents

Abstract Within the bio-economy, more specifically within the bio-based industries, biomass feedstock – in contrast to fossil feedstocks in the fossil-based economy – is converted into the so-called bio-based products such as biosurfactants, bioplastics, pharmaceuticals, paper, textiles, and biofuels using either chemical or biological production methods or a combination thereof. In Europe a turnover of 60 billion EUR is associated with bio-based plastics and chemicals such as biosurfactants, and 40% of the global biosurfactant market turnover is associated with the European market. The growing use of bio-based surfactants in detergents, personal care products, and oilfield chemicals is fueling the growth of this market, which is driven, in many applications, by more stringent regulations and by an increasing consumer demand for "green and sustainable" products. Microbial biosurfactants are a biologically produced type of bio-based surfactants which are quickly evolving from a scientific curiosity to an industrial reality.

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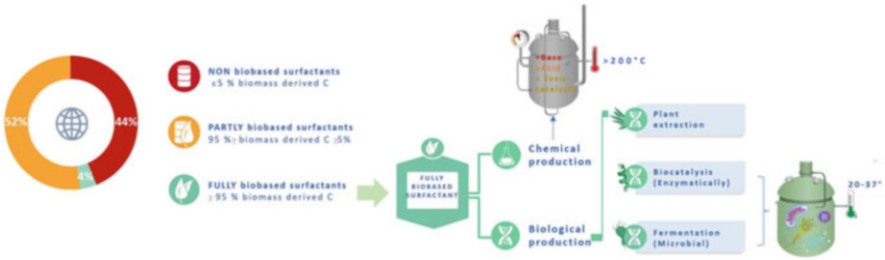
Graphical Abstract



Keywords Biosurfactant, Industry, Innovation, Investments, Market failure, Microbial biosurfactant

1 Introduction

Anno 2022, one cannot open a newspaper without reading about global warming, greenhouse gas (GHG) emissions, (plastic) waste and pollution, and the imminent shortage of petroleum in the future. Together this has resulted into a new and growing movement toward the development of a bio-based economy providing an alternative for the fossil-based economy. The feedstock used in the bio-economy is "biomass," which – in contrast to fossil feedstocks – is a renewable feedstock derived from land and sea: i.e., from plants, algae, animals, fungi, and microorganisms. Within the bio-economy, more specifically within the bio-based industries, this biomass feedstock is converted into the so-called bio-based products including biochemicals (e.g. biosurfactants), bioplastics, pharmaceuticals, paper, forest-based industries, textiles, biofuels, and bioenergy using both chemical and biological production methods. The percentage of bio-based carbon in chemicals and chemical products produced in the EU-28 – excluding biodiesel and bioethanol (NACE division 20) – corresponded to 15% in 2017. Surfactants are a class of chemicals that fall under the abovementioned category of chemicals and a big portion of the carbon used to produce surfactants is already derived from biomass (i.e., oleochemicals) while about 4% of the globally produced surfactants are fully bio-based [1]. The bio-economy (see Fig. 1) in the EU-28 was characterized by a turnover of 2.4 trillion EUR in 2017 of which 20% is associated with the primary sector producing the biomass feedstock, i.e. agriculture, fishery, forestry, etc. and 50% by its main downstream markets, i.e. the food and beverage industries. The remaining 30% of the overall European turnover (750 billion EUR) is generated by the abovementioned "bio-based industries" of which 60 billion EUR turnover is associated with bio-based chemicals and plastics under which category bio-based surfactants also fall.

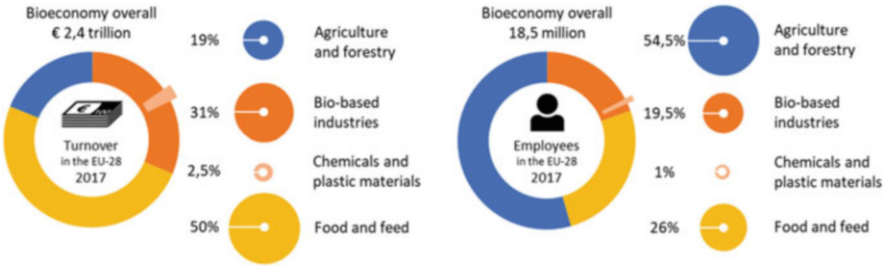


Fig. 1 Overall turnover and employment of the bio-economy and its bio-based industries in the EU-28 in 2017 (figure adapted from [2])

Throughout this chapter we will use the term "biosurfactant" as a synonym for "bio-based surfactant" or "wholly bio-based surfactant," which thus refers to the origin of the used carbon in these molecules (i.e., 100% bio-based), just as is the case for the term "bioplastic," which is synonymous for bio-based plastics. Wholly bio-based surfactants are surfactants of which the carbon is entirely (100%) derived from biomass as defined by the European Committee for Standardization – Technical Committee 411 (bio-based products) and 276 (surface-active agents). The use of clear and uniform terminology is extremely important as misuse and non-uniform use will result in confusion, not only in the field, but also with the consumer, which is highly undesired. In specialized literature about microbial biosurfactants, the term "biosurfactant" is often used as a synonym for "microbial biosurfactants," which according to the authors is confusing and not in line with other definitions used, e.g. bioplastics, biofuels, etc. where "bio" refers to the origin of the contained carbon herein. We will come back to this aspect later on.

The most significant part of the global bio-based surfactant or biosurfactant market are the chemically produced biosurfactants (see below) and this market is projected to reach USD 17.27 billion by 2022, at a CAGR of 5.1% between 2017 and 2022 [3], while the microbial biosurfactant market is projected to reach 5.5 million by 2022 [5]. The market share of chemically produced biosurfactants corresponded to about 30% in value of the total global surfactant market in 2017 [3, 4]. About 40% of the biosurfactant market turnover is associated with the European market [3, 5]. The growing use of bio-based surfactants in detergents, personal care products, and oilfield chemicals is fueling the growth of this market, which is driven, in many applications, by more stringent regulations and by an increasing consumer demand for "green and sustainable" products. Many companies also have incorporated sustainability as a core value into their "company goals and values," which also puts pressure from within to achieve them. The authors of this book chapter are convinced that companies not making this switch will not survive in this very fast changing landscape.

The focus of this book chapter lies on a specific type biosurfactants: the so-called microbial biosurfactants. These are biosurfactans which are produced by microorganisms, such as bacteria and fungi, and are in general divided into glycolipids;

lipopeptides; neutral lipids and fatty acids; phospholipids and polymeric biosurfactants. According to a recent market report, the global microbial biosurfactant market was valued at USD 3.99 Billion in 2016 and is projected to reach USD 5.52 Billion by 2022, at a CAGR of 5.6% during the forecast period in contrast to a CAGR of 5.0% for the fossil-based surfactants [5]. Similar to the overall market of bio-based surfactants, the major revenue share of the microbial biosurfactant market is associated with the European market with about 45% market share associated with Europe in 2016 [5]. According to the authors' information and according to ECCHA, the production volumes of the various types of microbial biosurfactants in Europe mentioned in this market report (200,000 tonnes in 2017) in reality remain modest (100–1,000 tonnes range in total in Europe), which is thus a factor 200 lower compared to the volumes mentioned in the abovementioned market report, which the authors of this book chapter thus doubt. Also, when looking at the mentioned market value, this would correspond to an average cost of 2 euro/kg of microbial biosurfactant, which is also at least a factor 10 off compared to reality. Although the values mentioned in this report are thus questionable to say the least, microbial biosurfactants are regarded as one of the top emerging bio-based products [6] and a significant increase both in volumes and revenues is expected the next few years as will be further discussed below.

2 Surfactants, Biosurfactants, and Microbial Biosurfactants

Surfactants are industrially important chemicals. The global surfactants market accounted to around 43 Billion dollars in 2017 and is estimated to reach 55 Billion dollars by 2027 [7]. In its totality surfactants are one of the largest markets (20 Million tonnes/year) of bulk chemicals. Surfactants are performance molecules that intervene in nearly every aspect of our daily lives due to their versatile surface-active properties. They are applied in consumer- and industrial products such as personal care, cosmetics, cleaning, paints and coatings, chemical industry, pharmaceuticals, textiles, softeners, food and feed, beverages, crop protection, metal extraction, bioremediation, packaging, construction, pulp and paper, etc. (see Fig. 2). The most important drivers of the growth of the surfactant market are a rising demand for personal care products and from the Asia-Pacific region, which are both expected to witness the highest growth rate. The implementation of stringent regulations by government agencies is one of its main restraints [4].

Looking at the sourcing of feedstock for surfactant production (see Fig. 3), the surfactant market can roughly be divided into three segments: non-bio-based surfactants (fully derived from fossil resources), partly bio-based, and fully bio-based surfactants. The partly bio-based surfactants can be further divided into majority ($95 \geq x > 50$) and minority ($50 \geq x \geq 5$) bio-based surfactants. As mentioned above, approximately 4% of the produced surfactants are fully bio-based, which means the

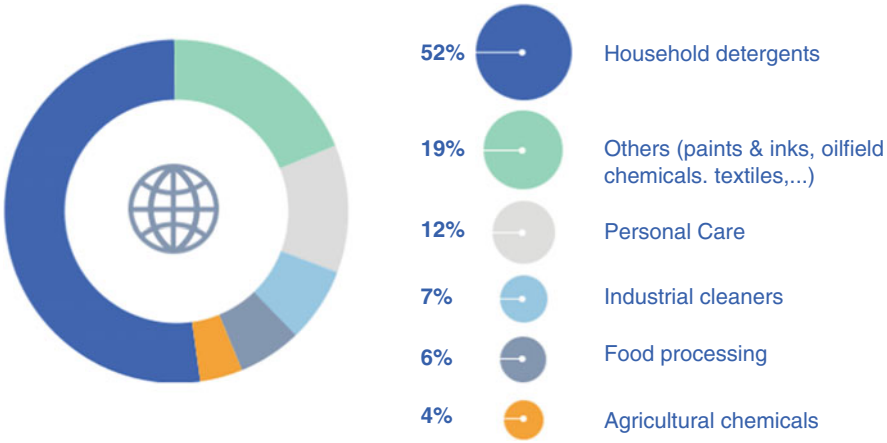


Fig. 2 Overview of the applications of surfactants. *The volume is composed of a low value detergent segment and a higher-value segment, which includes cosmetics, personal care, and other higher-end markets.* More than half of the produced production volume of surfactants is used for application in household detergents

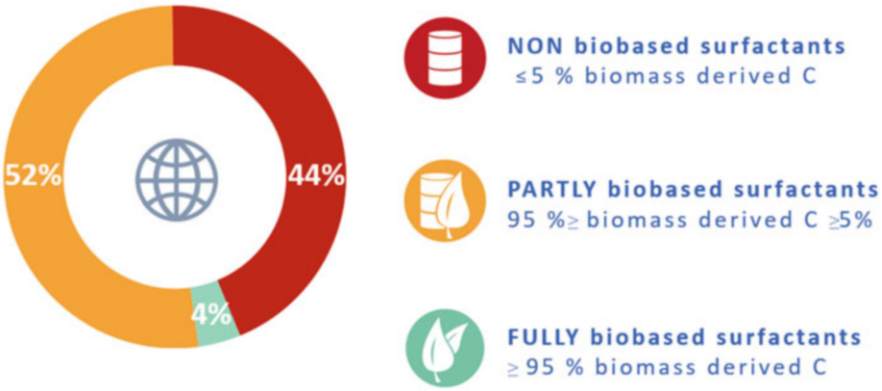


Fig. 3 Feedstock sourcing of the carbon contained within the total surfactant market. The largest part of the market (52%) is "partly bio-based," which comprises the "majority" ($95 \geq x > 50$) and "minority" ($50 \geq x \geq 5$) bio-based surfactants, while only a small portion is fully bio-based (about 4%). Still 44% of the market volume is fully fossil based

carbon contained within these surfactants is entirely derived from biomass as can be determined by C-14 analysis. An error margin of 5% is allowed.

Fully bio-based surfactants can be produced through *chemical or biological means* (see Fig. 4) and chemically produced biosurfactants currently dominate the biosurfactant market. One of the very first "surfactants" produced by humans, of which the production and use at least dates back to 2,800 BC [9], is a chemically produced biosurfactant, which we all know very well: "soap." Soap is produced

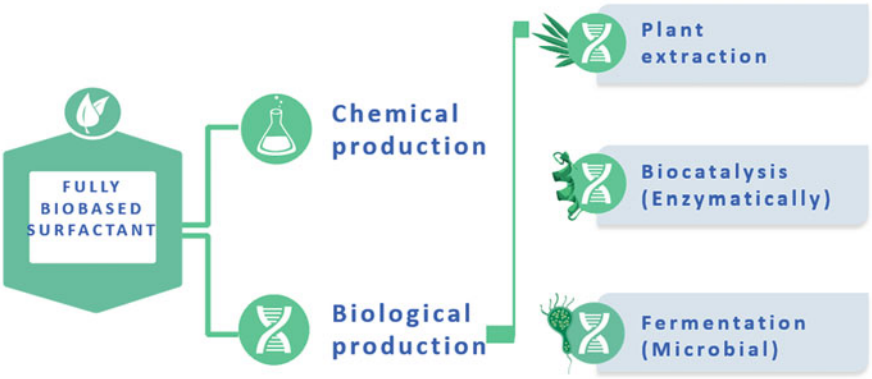


Fig. 4 Production methods for fully or wholly bio-based surfactants

using a very straightforward (one- or two-step) chemical process (saponification), converting vegetable oils or animal fats and lye (e.g., sodium hydroxide) into soap and glycerol. Soap (salts of fatty acids) are thus also bio-based surfactants, as the carbon contained within soap is entirely derived from animal and/or plant-derived biomass. The hydrophilic part contained within soap is thus an inorganic compound such as sodium, kalium, etc. combined with the carboxy group of the fatty acid.

In the last decades other more specialized *chemically produced biosurfactants* were developed and commercialized: Methyl Ether Sulfonates (MES), Alkyl Polyglucosides (APGs), sorbitan esters (Spans), sucrose ester, betaines, and fatty acid glucamides [3]. MES, APGs, and sorbitan esters currently have the largest markets and are produced by many different companies such as Stepan Company, Lion Corporation, Guangzhou Lonkey, BASF SE, Dow Chemical Company, Nouryon (Akzo Nobel), Kao Corporation, Croda International Plc, Seppic and Henkel KGaA. Sucrose esters or fatty acid glucamides are produced in smaller amounts, respectively, by, for example, Sisterna and Clariant AG. Croda recently has invested 170 million US dollars into a production plant for the production of fully bio-based ethylene oxide to be used for the production of 100% bio-based non-ionic bio-based surfactants [10].

The substrates for chemical biosurfactant production are typically refined substrates such as carbohydrates (e.g., sucrose, glucose, etc.) and fatty alcohols/fatty acids/oils. It should be mentioned that for the sourcing of the latter substrates, the market largely relies on palm oil derived from tropical areas for the provision of the hydrophobic moiety, which is associated with clear concerns. The chain length (short to medium chain length) required for good functionality of these surfactants is typically present in these tropical plant oils and not in plant oils from more moderate climates (mainly long chain lengths). Palm oil cultivation is characterized by high yields of oil per hectare, but as the available plantations could not cope with the increasing demand, this resulted in deforestation of pristine forest inhabited by endangered animals such as orang-outangs resulting in clear concerns associated

with palm oil production. It makes no sense to shift to other plant oils, such as coconut oil (which is sometimes advocated for as an alternative for palm oil) as the yields per hectare are lower, which would thus result in even more deforestation for the same amount of oil. The RSPO (roundtable on sustainable palm oil) was initiated in 2004 to make sustainable palm oil the norm. However, RSPO is criticized (e.g. by IUCN (International Union for Conservation of Nature and Natural Resources)) for only providing a marginal advantage in terms of putting a halt to deforestation compared to non-certified palm oil and is providing some suggestions on how to improve in the future [11]. Alternative/complementary solutions allowing circular and more sustainable production not relying on tropical oils as an alternative for fossil oils are thus sought for, which could be supplied by (some of) the examples provided below.

The second type of biosurfactants are produced through *biological production methods* and can be divided into three types: those extracted from plants, those obtained using enzymes (biocatalysis), and a last type obtained using microbes: microbial biosurfactants. The three types will be further described below.

One of the best-known examples of biosurfactants *extracted from plants* are saponins [12]. Saponins are a group of glycosides produced as secondary plant metabolites which are widely represented in the plant kingdom in levels ranging between 0.1 and 30%. Saponins were actually one of the very first surfactants used by mankind as "soap," which is also reflected in its name: the Latin word "*sapo"* means soap. Saponins are mainly commercialized by Asian (mainly Chinese) companies (e.g., Laozhiqing Group, Yongxin Youxiang, Tianmao, Hubei Jusheng Technology, and Weihe Pharma) [12] and are extracted from various plant materials (leaves, legumes, roots, flowers, etc.). The global saponin market increased from 13.3 kilo tonnes in 2011 to 14.7 kilo tonnes in 2016 with an average growth rate of 1.97% and was valued at US$ 954 Million in 2019 [12] and is slowly growing with a CAGR of 0.2%. Saponins are applied in cosmetic, food, agrochemical, and other applications for their physicochemical (surfactant) properties, but also for their biological activity (antimicrobial, anticholesterol, etc.) and/or the combination thereof [13]. The main substrate for plant derived biosurfactants is CO_2 taken up from the atmosphere by the plants and the cultivation of the plants also requires the use of fertilizers and water as important resources. The saponins are extracted from the plant materials using various solvent-based approaches.

Biocatalytic production of biosurfactants has until today not resulted in large-scale production volumes of biosurfactants. A few examples are commercialized, e.g. by Evonik, but the production volumes remain low. However, biocatalysis is a powerful and versatile tool to replace and/or complement chemical routes, especially toward the synthesis of biosurfactants, i.e. starting from biomass. One could image biocatalytic routes for the production of (some of) the abovementioned chemically produced biosurfactants: sugar esters, alkyl poly glycosides, etc. A number of biotech companies have biocatalytic platform technology available, which is suitable to move into this direction and are currently looking into opportunities while optimizing the technology. The authors of this book chapter thus expect this technology to lift off in the coming years as the "need for green" (see below)

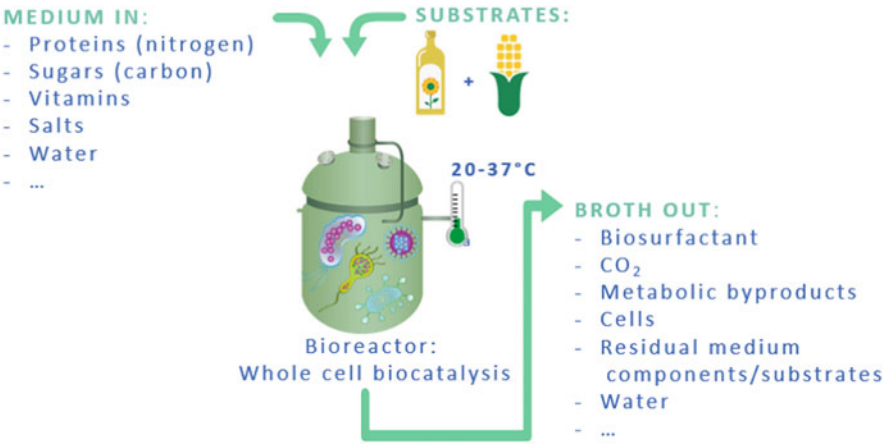


Fig. 5 Schematic representation of a production process for microbial biosurfactants. Typical processes are operated between 20 and 37°C at low pressures and in water. Medium components provide elements for growth and maintenance of the microorganism (typically in a monoculture) and substrates are fed for conversion into biosurfactants

increases and companies are increasingly and more urgently looking for greener production routes and products. However, similar bottlenecks and hurdles exist as for microbial biosurfactants, explained in detail below: further investments in the optimization and elaboration of this technology will be required to enable it to serve as base technology for cost-efficient and scalable production of (bulk) biosurfactants. The authors expect these technologies to lift off in the coming years. The substrates for biocatalytic production of biosurfactants are typically the so-called first-generation refined substrates such as carbohydrates or amino acids and fats and oils.

The third type of biologically produced biosurfactants are *microbial biosurfactants*. Here microbes such as fungi, yeasts, and bacteria are used as biocatalysts to produce biosurfactants (Fig. 5).

A wide range of biomass substrates can be converted into biosurfactants by different types and species of microorganisms through the complex biocatalytic pathways encoded within their genomes. This is associated with certain benefits, as these technologies do not require the use of first-generation refined substrates (although these are currently used for industrial production for practical reasons) such as refined sugar and/or oils/fatty acids, but allow the use of waste- and/or side streams, e.g. from (industrial) food waste and/or agro- origin. This opens up opportunities for circular and more sustainable production of biosurfactants as the use of first-generation substrates such as glucose and plant oils has a significant negative effect on the sustainability of microbial biosurfactants as demonstrated for sophorolipids [14]. Moreover, some microorganisms are capable of producing fatty acids, fatty alcohols, etc. for incorporation in biosurfactants starting from other types of (lower cost, lower impact) carbon sources, such as (waste)

carbohydrate streams, e.g. food waste to SLs [15], glucose to RLs [16], etc. Both SLs and RLs contain a lipophilic part incorporated in their structures. The microorganism thus uses its metabolic pathways to convert the carbohydrate into a lipophilic precursor for conversion into a biosurfactant. This omits the need to supply certain higher cost or higher impact (to the environment) substrates (e.g., fatty alcohols) into the process, which again represents a serious advantage. It should be noted that the above is only true in case the process efficiencies are similar. The latter aspect is often forgotten by the microbial biosurfactant community. Lower cost and lower impact substrates can seem very interesting, but if the process efficiencies drop significantly, this will obviously negatively affect the overall costs and sustainability of the biosurfactant. This can result in an even higher cost and higher impact per kg of produced biosurfactant compared to the reference (in this case a first-generation biosurfactant), but is often not considered by researchers looking into alternative substrates. Moreover, the variability and availability at the relevant scale of such alternative substrates should be taken into account by researchers early on. Often marginal, highly scattered and variable streams are considered in publications, without taking into account the projected production scales, logistics (often impossible) or variability within these biomass streams. A higher sense of responsibility and interaction with the "real world" is thus required from academic researchers to investigate and define solutions which are relevant to the "real world" and thus to the industry and not just nice to publish.

Many of the microbial biosurfactant technologies are still immature and operated at small scale (lab to 100 tonnes range) resulting in high costs compared to traditional surfactants as the economy of scale has not kicked in yet [17], but also compared to chemically produced biosurfactants such as APGs, which are already more expensive compared to fossil derived biosurfactants. The higher costs compared to chemically produced biosurfactants from similar resources can generally be attributed to three main reasons according to the authors: scale, immaturity of processes (not optimized yet), and typically new installations required (new CAPEX), which is not always the case for chemical processes for which many plants exist with some spare capacity and (partly) depreciated installations.

The field of commercialized *microbial biosurfactants* is thus still quite limited in types and producers/suppliers. Summarized, the commericalized products are *glycolipids* (sophorolipids (SLs), rhamnolipids (RLs), and mannosyl erythritol lipids (MELs)), *lipopetides (LPs)*, *phospholipids/fatty acids,* and *particulate* and *polymeric biosurfactants*. About 10 companies worldwide are producing and commercializing microbial biosurfactants of which SLs take the biggest market share followed by RLs, MELs, and LPs [3]. An increasing amount of B2B and B2C companies is taking interest in microbial biosurfactants in general, which the authors of this book chapter clearly notice by the amount of new parties expressing their interest in the field through the extensive network the Bio Base Europe Pilot Plant and InBio.be.

Sophorolipids produced by *Starmerella bombicola* are commercialized by B2B companies such as Evonik, Wheatoleo, and Holiferm in Europe and applied in consumer products by several companies e.g. Ecover, Soliance, Henkel, etc. mainly for applications in household detergents and personal care products, but applications

are also emerging in agriculture, poultry farming, etc. Applications are also possible in the agrochemical, food, and pharmaceutical industry. A number of Asian companies also commercialize SLs, mainly for oilfield applications, but also for household and cosmetic applications and a number of Asian companies apply SLs in consumer products, e.g. Saraya. Companies in North- and South America are also investing in this technology and/or putting SLs on the market, e.g. Locus in the USA. The fact that SLs were the first microbial biosurfactant to really find their way to the market in a number of applications is because the natural SL producers, such as *S. bombicola*, are highly efficient microbial biosurfactant producers and productivities between 2 and 4 g/L h are reached, which are acceptable levels for industrial production.

Also, *rhamnolipids* are in the lift. Although RLs produced by *Pseudomonas aeruginosa* have been available on the market for over 20 years, e.g. commercialized by Jeneill and Rhamnolipid Inc., more recently other companies with larger ambitions have invested in production technology for rhamnolipids. The RL technology from Logos Technologies (NatSurFact) has recently been acquired by Stepan [18] and is currently producing at pilot scale. Evonik has taken a more surprising though very important approach. They developed a heterologous non-pathogenic (in contrast to *P. aeruginosa*) production host for RLs and are exploring the market together with Unilever. The RL containing dishwash liquid Quix was launched on the market by Unilever [19] and received with big success. The construction of a multi-thousand tonne production plant for rhamnolipids is currently ongoing based on the Evonik technology. This is a very important step in the further development of the microbial biosurfactant market. It shows that it is feasible to develop sustainable though cost-efficient and scalable biological production methods for commercially relevant and industrially scalable microbial biosurfactants. Evonik had already set a milestone in 2015 by launching SLs on the B2B market (Rewoferm), and now, only a few years later they launch a second microbial biosurfactant on the market. This has been received as big and important news in the surfactant market to say the least. Also, this success story indirectly makes a very clear statement about the use of GM (genetically modified) derived products in consumer products. The reason therefore is the fact that the most efficient wild-type RL producer *P. aeruginosa* is an opportunistic pathogen rendering large-scale industrial production of RLs with an efficient wild-type organism troublesome. Although heterologous production of RLs seems an obvious option, this has been an important bottleneck in the past decade mainly through negative public perception associated with GMO derived products.

Clearly the consumer comes in contact with many GMO derived products (not containing the genetically modified organism itself) on a daily basis (e.g., enzymes used in detergents, insulin, enzymes used in the food industry, novel food products, amino acids, etc.). However, consumers are typically not aware of this fact and at the same time very sceptic about GM technology. The term "GMO" has a negative connotation, because the consumer in general is not well informed about this topic and a lot of Scepticism and suspicion exists. However, biotechnology and GM derived products are key toward the transition of a fossil-based to a bio-based

economy. In the past, big companies often stayed away from GM technology for the production of ingredients for consumer products such as household detergents and cosmetics due to potential negative effects exerted through public perception on their businesses. However, increasing sustainability and moving away from fossil-based technologies inevitably requires the use of molecular biology and GM organisms and catalysts. The abovementioned joint venture can thus be considered as a very important step in the good direction and according to the authors will be a milestone in the field of microbial biosurfactants as well.

Lipopeptides are a quite diverse group of microbial biosurfactants with surfactin being the best-known one. The commercialized lipopeptides are typically produced with *Bacillus* species, e.g. Lipofabrik. Although these are very promising molecules and strong surfactants, the production volumes remain low. This is mainly because the natural production capabilities of the producing microorganisms are typically a factor 10–20 lower compared to those obtained for SLs and RLs, which makes the compounds quite expensive. However, lipopeptides are – similar to saponins described above – besides their surfactant properties also characterized by dual functionality, e.g. strong antimicrobial effects, elicitation effects in plants (arousing defense mechanisms before plant pathogens are present, as such making the plants more resistant against pests: hence the name "plant vaccination"). These non-surfactant properties have already resulted in market introduction of lipopeptides in agricultural applications and post-harvest protection, e.g. by Janssen PMP.

Mannosylerythritol lipids have been the subject of much industrial interest during the past few years, many companies have looked into production technology for MELs, e.g. Oleon, Fraunhofer, etc. However, MELs are quite complex compounds with many variants being co-produced resulting in a quite complex production profile, which is influenced by a variety of factors and not easy to control. Although clear interest in these compounds in the industry thus exists, further optimization and investigation is required to develop robust and scalable processes. Oleon is planning to produce and market MELs in the near future.

3 The Trend for Biosurfactants

Biosurfactants and especially biologically produced biosurfactants have recently attracted strong attention from surfactant producers because the consumer demand for greener products has gone mainstream. In contrast to a decade ago, a large part of the general public – especially in the Western world – is aware of and acknowledges the problematics associated with pollution, global warming, and deforestation. Although the shift toward a bio-based economy is inevitable toward sustaining mankind's future on earth, the only factor that has really forced true and significant change in the past is (changing) legislation imposed by national and international governments. This is, according to the authors, also expected to remain the most powerful method for change. However, in this digitalized society, the influence of

the consumer cannot be underestimated. Social media have provided consumers with a very powerful tool to effectively criticize (large) companies about aspects such as sustainability, feedstock sourcing and safety, which was not the case in the past. The rise of social media also assisted in the abovementioned increasing awareness, although it also results in polarization.

The drive toward more sustainable products is now thus finally moving into the realization phase. Most industrial companies are currently expanding/initiating their bio-based portfolio and investing in sustainability not to fall behind. This resulted in a clear increase in R&D developments of bio-based alternatives for both commodity and high-value molecules over the past few years. However, as R&D and innovation require time and money, there currently exists a disbalance between the demand and supply for biosurfactants: biosurfactants are currently not available at large scale at affordable cost, which is in sharp contrast to the clear demand. This market failure is not so easy to overcome as the risk of venturing into the biosurfactant field is still quite high for industrial companies. Moreover, most conventional surfactant producers are chemistry based, they are not familiar with bioprocessing and typically have no bioproduction facilities. The result is that many conventional surfactant producers are now actively looking for industrial manufacturing technology for biosurfactants. This demand for industrial production technology is currently not met as most of the microbial biosurfactant technologies are still immature. However, the abovementioned recent successful venture of an established "traditional" surfactant producer, i.e. Evonik into the microbial biosurfactants area has demonstrated that biosurfactants are quickly evolving from a scientific curiosity to an industrial reality. The pivoting point and shift in ambitions from the industry thus seems to be reached. A clear proof of this eminent switch was provided by a Unilever's recent statements toward their ambition to completely eliminate fossil feedstocks for the production of cleaning products by 2030 [20] and a reserved budget of one billion euro to do so. Their abovementioned investments together with Evonik to build a multi-thousand tonne scale microbial biosurfactant (rhamnolipid) production plant in Europe are clearly a building block in this strategy. The substantioal investments these two companies are making is backed up with a clear market pull of consumers for green and mild products, which was shown by the great success of the RL based liquid detergent QUIX. As Unilever is one of the largest brand owners, this puts large pressure on B2B companies supplying ingredients and materials to Unilever.

4 Opportunities and Restraints for (Microbal) Bio-surfactants

The surfactant market grew at a CAGR of 5.4% over the past 5 years [4]. The most important drivers of the growth of the surfactant market are a rising demand for personal care products and from the Asia-Pacific region, which are both expected to witness the highest growth rate. The implementation of stringent regulations by

government agencies is one of its main restraints. The main current market opportunity is the production of sustainable green surfactants [3, 4]. The chemically produced biosurfactant market grew at a CAGR of 5.1% over the past 5 years, while the microbial biosurfactant market grew at a CAGR of 5.6% during the same period [3, 5]. This faster growth rate demonstrates the opportunity and demonstrates the potential impact of this technology on the market.

Especially the cosmetic and personal care industry are now serious about making the switch to bio-based ingredients and biosurfactants are an important developing field within the abovementioned megatrend. The personal care segment is estimated to experience the largest growth (CAGR of 5.7%) of all market segments [3]. The main reasons for the expected growth are the big demand in the growing moisturizing cream industry due to rising skin concerns among consumers. Biosurfactants show interesting properties for a more widespread implementation in skin cosmetics. Interesting target markets for market entry of novel (microbial) biosurfactants are thus the personal care and cosmetics markets, which are the second largest application sectors of surfactants after detergents. This market segment is characterized by (1) a higher accepted average cost of the used surfactants and (2) a clear continual pressure from consumers based on "green" factors, climate change, carbon footprint, deforestation linked to palm oil, but also mildness and undesired activities linked with preservatives in such products. Moreover, surfactants are the largest ingredient category within the personal care segment (25–30% share) [8]. A shampoo, for example, can contain up to 20 different types of surfactants. The abovementioned market segments are as mentioned above very susceptible to green and mild alternatives to "traditional" surfactants. Moreover, some (microbial) biosurfactants are also characterized by additional properties such as antimicrobial properties, which also holds a clear opportunity in these markets where several players are looking to decrease the use of preservatives in their products and are in need of safe and sustainable alternatives.

However, once a novel biosurfactant has been developed to a point that commercial application in, e.g. skin care products becomes relevant, still a large amount of work lies ahead as the reformulation of personal care products with new ingredients requires time and money. The *high safety standards in* this industry in conjunction with the banning of animal testing leads to the current situation that the desire for change is clear but the actual change is rather slow. Moreover, for most of these novel technologies the current costs are as mentioned above a factor 10–50 more expensive compared to conventional bulk surfactants. Bringing the technologies to larger scale would result in lower costs through the economy of scale and through further optimization. However, the high current costs inhibit a lift off of the market and production and selling volumes. One of the solutions to escape from this catch-22 situation is to invest in larger scale production capacities besides investing in optimization of production technology to increase the efficiencies. Especially for the first solution a clear ambition from the industry is required. Such ambition has clearly been shown as mentioned above by Evonik-Unilever, Stepan, and more recently also BASF [21] and more examples are expected to follow now that the tone is set and clear actions are demanded by the consumer.

5 Ambitions of the Authors

The authors of this book chapter have been working on a microbial biosurfactant platform technology at UGent (InBio.be) and BBEPP over the past 15 years. Several novel microbial biosurfactants were developed and scaled up to 15 m^3 scale. Within the framework of several (international) projects a vast network of parties active in the surfactants value chain has been built and new collaborations were setup. This allows us to state that a clear traction has been building up toward (microbial) biosurfactants. The authors are planning to valorize the developed technology by setting up the spin-off company "Amphi-Star" This spin-off aims to accelerate the generation of variation on the (microbial) biosurfactant market and would be complementary to the already commercialized compounds. The main hurdles to overcome are related to cost, registration, and further upscaling to large industrial scale. Given the low maturity of the microbial biosurfactant market, a technology package will be offered to the industry as the most attractive valorization path for the developed technology.

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Screening Strategies for Biosurfactant Discovery

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Contents

Abstract The isolation and screening of bacteria and fungi for the production of surface-active compounds has been the basis for the majority of the biosurfactants discovered to date. Hence, a wide variety of well-established and relatively simple methods are available for screening, mostly focused on the detection of surface or interfacial activity of the culture supernatant. However, the success of any biodiscovery effort, specifically aiming to access novelty, relies directly on the

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characteristics being screened for and the uniqueness of the microorganisms being screened. Therefore, given that rather few novel biosurfactant structures have been discovered during the last decade, advanced strategies are now needed to widen access to novel chemistries and properties. In addition, more modern Omics technologies should be considered to the traditional culture-based approaches for biosurfactant discovery. This chapter summarizes the screening methods and strategies typically used for the discovery of biosurfactants and highlights some of the Omics-based approaches that have resulted in the discovery of unique biosurfactants. These studies illustrate the potentially enormous diversity that has yet to be unlocked and how we can begin to tap into these biological resources.

Keywords Bioprospecting, High-throughput, Functional properties, Microbial, OMICS, OSMAC, Sequence-based, Surface-active

1 Introduction

Diverse microbial genera, from all domains of life, Bacteria, Archaea, and Eukaryota, have been identified as biosurfactant producers. To this end biodiscovery efforts have primarily focused on the screening of culturable isolates and generally included (1) the enrichment of microorganisms from a wide range of environments; (2) high-throughput screening using numerous assays suitable for the evaluation of large numbers of isolates; followed by (3) isolation and structural determination of the biosurfactant (Fig. 1). To increase the chance of identifying a novel compound, several culturing considerations have been applied in the biodiscovery stage. These include sampling from exotic (extreme) and underexplored environments [1]; targeting environments with prior exposure to hydrocarbon pollutants which have naturally selected for biosurfactant-producing microorganisms [2–4]; utilizing hydrophobic compounds in the culture media to enrich for the most capable producers [5]; and focusing on microorganisms from underrepresented phyla [6]. Considering that the compound identification and structural determination entails a time-consuming investigation, strain prioritization and dereplication are normally applied after the primary screening. Factors that are typically considered to select the most superior and novel producers include the novelty of the strain, the biosurfactant yield (based on the initial screening), and the activity range across property-based assays [7]. However, despite all these considerations, the structural diversity of the commercially available biosurfactants remains limited, and literature continues to report the rediscovery of structurally similar biosurfactants. Therefore, to identify truly novel biosurfactants, discovery efforts need rejuvenated approaches that critically assess the aspects that limit access to the novelty that is sought.

Indicated by Omics studies, it has become widely accepted that we have only scraped the tip of the iceberg in terms of accessing the biotechnological potential harbored in microorganisms. It can be reasonably expected, therefore, that there is yet much biosurfactant diversity to be discovered by investigating non-cultivable

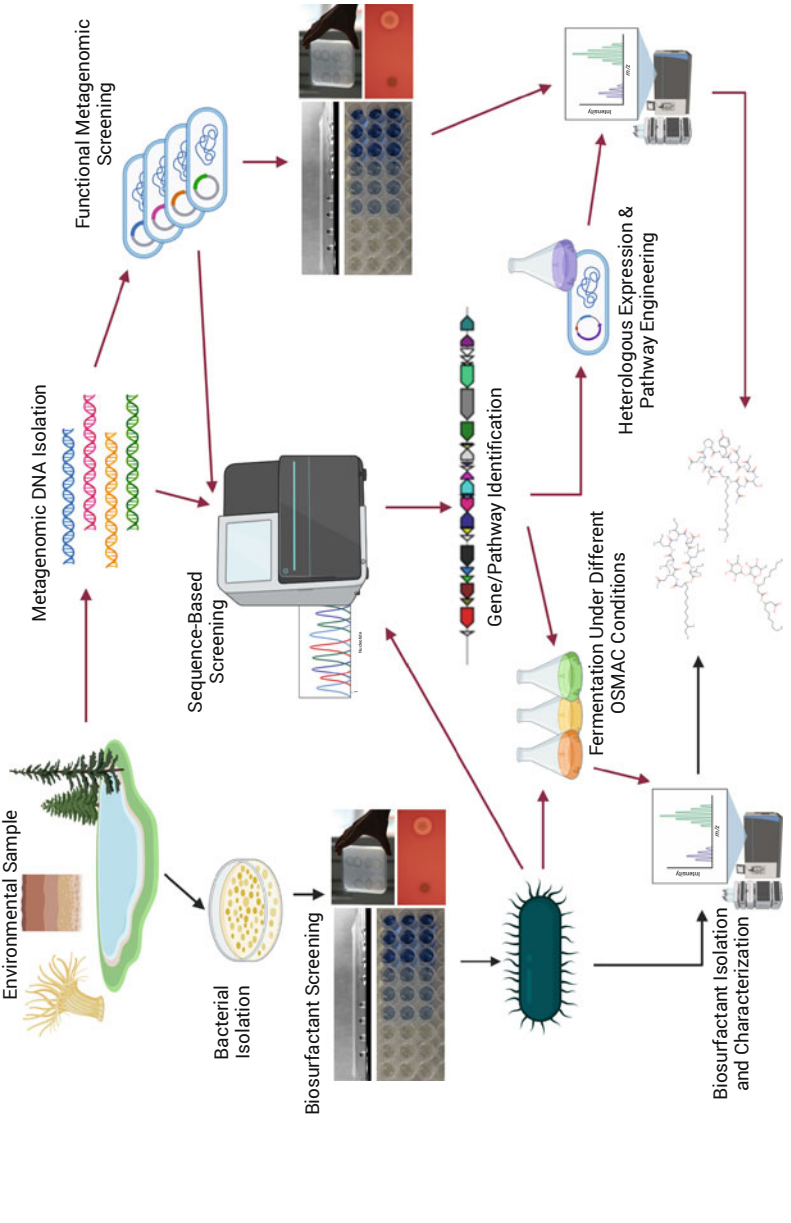

Fig. 1 The biosurfactant discovery pipeline. Biosurfactant discovery has largely been conducted through the isolation and screening of bacteria and fungi using traditional microbiological techniques (black arrows). Given that rather few novel biosurfactant structures have been discovered during the last decade, advanced strategies are needed to widen access to novel chemistries and properties. Omics technologies (red arrows) are beginning to yield unique biosurfactants and offer powerful tools to explore the expansive potential harbored in natural environments

microorganisms using culture-independent screening approaches, followed by the assessment of the activity through heterologous expression (Fig. 1). Moreover, the increased sequencing of culturable bacterial genomes has revealed great disparity between the number of putative secondary metabolite gene clusters identified based on bioinformatics analyses vs. the actual number of secondary metabolites detected from culture fermentations [8]. This not only points to an astounding level of as yet undiscovered metabolites for the majority of cultured organisms, but it also provides the opportunity to employ sequence-based analyses to inform on strategies to elicit the biosynthetic pathways that remain silent during the growth of culturable isolates under standard laboratory conditions. The level to which genome mining (vs. screening for biosurfactant activity) can already contribute to biosurfactant discovery will be discussed in this chapter (Fig. 1).

Regardless of whether culture-based or culture-independent approaches are employed for biosurfactant discovery, assessment of the activity constitutes a crucial component of each biodiscovery approach. Focused on the detection of novel biosurfactants with specific physicochemical characteristics and performance properties, the selection of a strategy to screen for biosurfactant-producing microorganisms is of vital importance. The strategy sets the scene for the likelihood and the level of success in the identification of novel and structurally diverse biosurfactants with potential commercial value in different sectors of bioeconomy. In the interest of product development, the strategy should not only incorporate methods to screen large collections of microorganisms rapidly, easily and with a high level of sensitivity, but should also facilitate the detection of specific biosurfactant characteristics and the quantification of key parameters for the selection of the most promising biosurfactant candidates. Therefore, this chapter will provide an exhaustive summary of the wide range of screening methods available, addressing specific factors that need to be considered and revised in the prospecting efforts to ensure that novel properties and structures are identified. Another major obstacle to the development of biosurfactants for commercial application is the yield, which may be factored into the screening process. However, some bioengineering approaches to improve strain yields are available, therefore, this chapter will not incorporate yield as a screening criterium.

2 Screening Methods Based on Physical Properties

A wide range of screening methods, based on the physicochemical properties displayed by surfactants, are available for the identification of biosurfactants produced by microorganisms. These are mostly dependent on direct and indirect measures of surface and interfacial tension activity, with a few methods that assess specific physicochemical features of specific groups of biosurfactants. Screening for surface activity, using a single or a combination of assays, can be performed on purified biosurfactants, on whole cells, or culture supernatants, where both qualitative and quantitative data can be obtained. The principal aim in such screenings is to

identify structurally new biosurfactants with effective surface and interfacial tension reduction, low critical micelle concentration (CMC), high emulsion capacity, good solubility and retained activity at a broad pH, salinity, and temperature range [10]. Notably, the screening methods can also be employed to prioritize cultures that could offer a commercial competitiveness by demonstrating a high production rate/yield at low production costs.

This section provides an overview of all the methods reported to date and highlights the screening strategies most often employed. The screening methods are categorized here as "Universal" – assays for the initial screening of large numbers or collections for general biosurfactant activity; or, "Targeted" – which includes either indirect assays to detect specific biochemical or performance properties or the more sophisticated direct assays to identify biosurfactants based on physicochemical properties. The described methods are compared in terms of necessary processing efforts, skill level, and equipment requirement, and suitability for high-throughput screening and quantification (Table 1). Notably, many of these methods lack specificity, therefore the initial results, in particular from universal screening methods, should be confirmed with complementary methods up to structural elucidation. A guideline for screening and characterization approaches for biosurfactants was recently suggested by Twigg et al. [29].

2.1 Universal Screening Assays

Included in this category are screening methods based on an indirect measure of the ability to reduce surface and interfacial tension, the phenotypic characteristics shared by surfactants. They are easy, rapid, sensitive, do not normally require expensive equipment (Table 1), and are often suitable for the screening of large numbers of cultured strains or metagenomic library clones. Hence, such assays are typically used as the first line of screening for the isolation of positive biosurfactant-producing strains/clones. Of all the assays described hereafter, the drop collapse, oil spread test, and the atomized oil spray have been the most widely applied in biodiscovery and screening studies [30, 31].

2.1.1 The Drop Collapse Test

This method assesses the stability of drops of culture broth, culture supernatant or solutions of pure biosurfactant when they encounter an oil-coated or hydrophobic surface (Fig. 2) [32]. The underlining principle is that the drop collapse occurs as a result of the reduction of interfacial tension within the liquid and the surface tension reduction on the hydrophobic surface caused by biosurfactant activity [33, 34]. The control drops without biosurfactant activity remain stable and do not collapse due to the hydrophobic surface repelling the polar water. The stability of the drop depends

Table 1 Comparison of the ease of use of the methods available for the screening of biosurfactants[a]

Screening method	Sample type	Sensitivity	Level of difficulty	Analysis speed	Special equipment required	Qualitative (QL), semi-quantitative (S-QN), quantitative (QN)	Applicability for high throughput screening [a]Can be adapted to be	Recent biodiscovery examples employing the respective method
Atomized Spray Method	Colonies	H	Easy	Sec	N	S-QN	Y	[11]
Drop Collapse Test	Culture supernatant, relatively pure compound	M	Easy	Min	N	S-QN	Y	[7]
Oil Spread Test	Culture supernatant, relatively pure compound	H	Easy	Sec	N	S-QN	N	[12]
Micro Plate Assay	Culture supernatant, relatively pure compound	H	Easy	Min	N	QL	Y	[13]
Penetration Assay	Culture supernatant	M	Easy	Min	N	QN	Y	[14]
Tilting Slide Test	Culture supernatant, relatively pure compound	M	Easy	Min	N	QL	N	[15]
VPBO-Assay	Culture supernatant, relatively pure compound	H (>CMC)	Easy	Sec	N	QN	Y	[16]
EC 24	Culture supernatant	M	Easy	Hours	N	QN	N	[13]
Hemolytic Assay	Colonies	M	Easy	Days	N	S-QN	N[a]	[7]
CTAB-Methylene Blue	Colonies	H	Easy	Days	N	S-QN	N[a]	[17]
CPC-Bromothymol Blue	Culture supernatant, crude extract	H	Easy	Min	N	S-QN QN	N[a] depends on the need for extraction	[18]
TLC	Crude extract	H	Medium	Hours	N	S-QN	N	[19]
Hydrocarbon Overlay Agar	Colonies	M	Easy	Days	N	QL	N	[20]
BATH	Culture with cells	H	Easy	Hours	Y	QL	N	[20]
HIC	Relatively pure compound	H	Challenging	Hours	Y	QL	N	[21]
Replica Plate Test Assay	Colonies	H	Easy	Min	N	QL	N	
Salt aggregation	Culture supernatant	H	Easy	Min	N	QN	N	[22]
Solubilization of Crystalline Anthracene	Culture supernatant, relatively pure compound	H	Challenging	Min	N	QN	N	
MALDI-TOF/MS	Crude extract	H	Advanced	Hours	Y	QN	N	[23]
Du-Nouy-Ring Method	Culture supernatant, relatively pure compound	H	Medium	Hours	Y	QN	N	[24]
Wilhelmy Plate Method	Culture supernatant, relatively pure compound	H	Medium	Hours	Y	QN	N	[25]
Stalagmometric Method	Culture supernatant	H	Medium	Hours	Y	QN	N	[26]
Axisymmetric Drop Shape Analysis by Profile	Culture supernatant, relatively pure compound	H	Challenging	Hours	Y	QN	N	[27]
Pendant Drop Shape Technique	Culture supernatant, relatively pure compound	H	Medium	Hours	Y	QN	N	[28]

[a]The methods are compared in terms of necessary processing efforts, skill level, and equipment requirement, and suitability for high-throughput screening and quantification. The methods are presented in four categories (yellow) "Universal" preliminary screening assays; (gray) methods that screen for specific biochemical or physicochemical characteristics and performance of the biosurfactant; and (green) those that directly measure surface and interfacial tension reduction

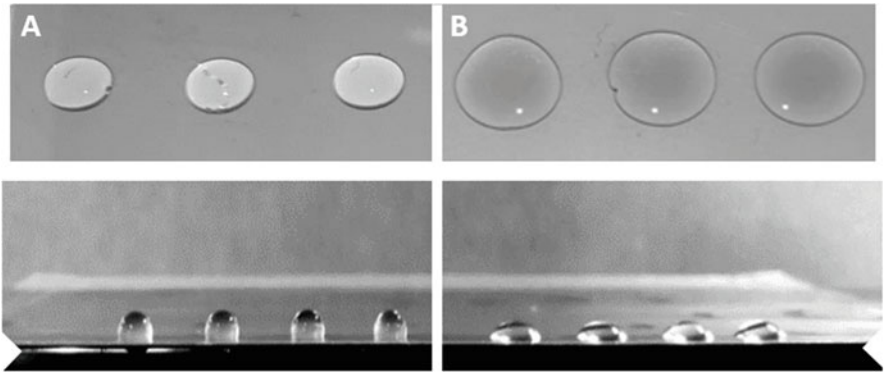

Fig. 2 The drop collapse method is used to screen for biosurfactant activity by observing the stability of drops containing biosurfactant on a hydrophobic surface. (**a**) Pure water samples without the presence of biosurfactant. (**b**) Mono-rhamnolipid containing supernatants of recombinant *P. putida*

on the concentration and surface tension reduction capabilities of the biosurfactant being screened.

One of the advantages of this method is its sensitivity and ease of use, with just a small volume of sample needed and no special equipment required [34]. It can be applied in large sample screening, and modifications to enable automated screening in microplates for high-throughput screening have been successfully employed [33, 35]. Staining of the droplets is an additional modification to enhance the visualization of the drops [36]. Disadvantageously, hydrophobic biosurfactants, and those that possess surface reduction capabilities but do not necessarily result in droplet collapse, cannot be detected using this method [37]. Activity relies obviously on a detectable concentration of the compound in the droplet and the hydrophobicity of the applied surface. Notably, there is actually no conclusive evidence that links surface tension reduction to the method, thus reducing its reliability. Lastly, the method is only qualitative, although it can be adapted to be quantitative for relative measurement of biosurfactant concentration for pure surfactants by measuring the drop size or the contact angle [7].

2.1.2 Oil Spread Test

The oil spread test is a rapid and easy method that does not require any special equipment [38]. A volume of crude oil is added to the surface of distilled water in a petri dish, resulting in the formation of a thin layer of oil on top of the water. The culture or culture supernatant is then placed on the oil layer, and where a biosurfactant is present, it displaces the oil creating a clear zone. This occurs as a result of the pressure formed upon contact of the hydrophobic part of the oil and that of the biosurfactant; the interface tension is reduced and the oil layer breaks resulting in a zone of clearing. The assumption is that the displacement of oil is directly

proportional to the concentration of the biosurfactant in the sample tested, and therefore provides both qualitative and semi-quantitative measurements [39]. A correlation between the oil spread test and the drop collapse method suggests that both could be used for preliminary screening; however, due to the disadvantages associated with the drop collapse method, perhaps the oil spread test represents a more appropriate assay for generalized screening.

2.1.3 The Atomized Oil Spray Method

In this assay a thin mist of paraffin is sprayed over bacterial colonies cultivated on agar plates, revealing the formation of droplets as a halo around a biosurfactant-producing colony (Fig. 3), the radius of which can be measured for a semi-quantitative analysis [35]. The method rapidly detects activity and does not require sample preparation, making it suitable for high-throughput screening of thousands of colonies at once, and therefore ideal for metagenomic library screening [31, 40]. Furthermore, the atomized spray method (also known as the oil vaporization assay)

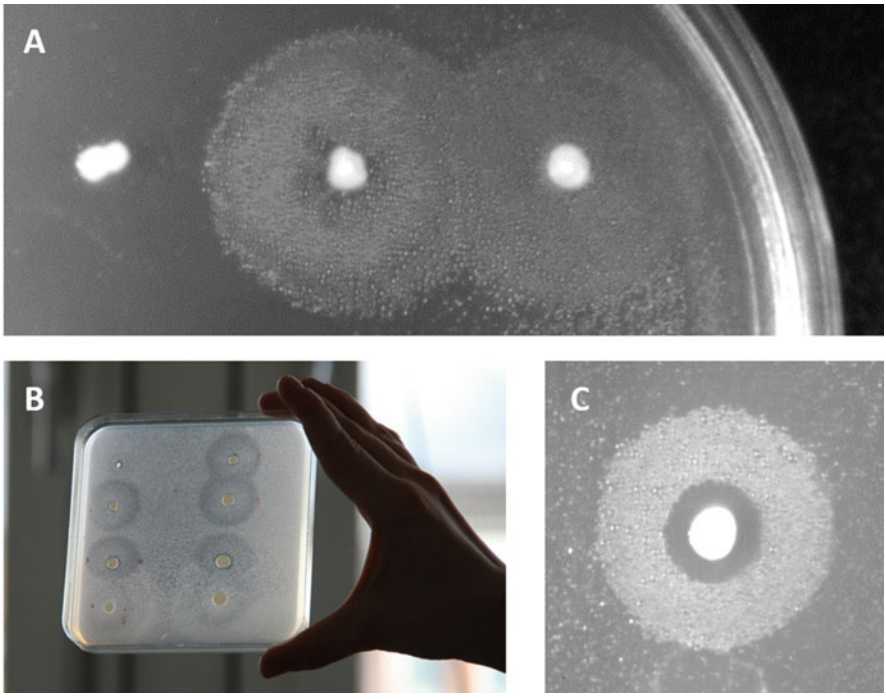

Fig. 3 The atomized oil spray method showing biosurfactant activity as a light-diffractive halo via formation of uniformed droplets surrounding microbial growth on agar. (**a**) Recombinant *E. coli* producing Serrawettin W1; (**b**) Recombinant *P. putida* producing mono-rhamnolipid; (**c**) Recombinant *Erwinia billingiae* producing Serrawettin W1

provides greater sensitivity than the drop collapse method, detecting 10–100-fold lower surfactant concentrations and allows the detection of biosurfactants with low water solubility [35]. Due to the versatility and sensitivity of the atomized oil assay, it is considered a more superior screening method than most of its indirect screening method counterparts.

2.1.4 Microplate Assay

The microwell plate assay has been patented as a qualitative measure to screen culture supernatant for surface tension reduction abilities [41]. The assay involves assessing optical changes of gridded paper placed under the 96 well plate containing the culture supernatant being tested (Fig. 4). Pure water in a hydrophobic well has a flat surface and no optical distortion, whereas the presence of a biosurfactant results in optical distortion. As a consequence, the fluid changes the surface brought about by the wetting of the edge of the well, subsequently becoming concave and taking the shape of the diverging lens. The assay is easy, rapid, sensitive, allowing instantaneous detection of surface-active compounds from a small volume [42] and is suitable for automated high-throughput screening, therefore appropriate as a method for functional metagenomic library screening [31]. Proper imaging is required to capture the correct optical distortion to remove subjective bias. Besides, the sample must be clear with no turbidity or intense color to observe the underlying grid changes [33].

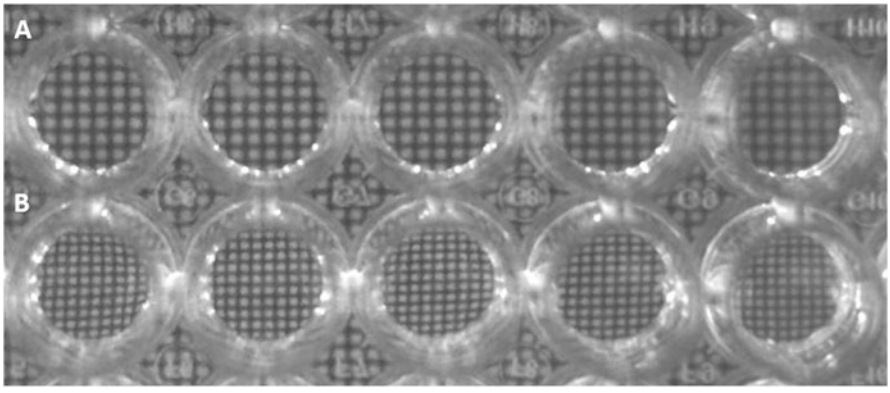

Fig. 4 Microwell plate assay measures the surface activity of biosurfactants in a solution. (**a**) Pure water in a hydrophobic well which has a flat surface; (**b**) optical distortion that is caused by mono-rhamnolipid recombinantly expressed in *P. putida*

2.1.5 Penetration Assay

This colorimetric qualitative assay is based on the infusion of two insoluble phases in a 96 well plate that results in a color change [43]. A hydrophobic paste mixture consisting of oil and silica gel applied to the wells is then covered with oil. The culture supernatant or sample, colored by adding a red staining solution, for example 1% safranin, is then placed on the surface of the oil mixture. In the presence of biosurfactant, the hydrophilic liquid breaks through the oil film barrier into the paste causing the stain to be absorbed by the silica, and the color of the upper phase changes from clear red to cloudy white within 15 min. The effect is based on the ability of the silica gel to enter the hydrophilic phase from the hydrophobic paste much more quickly in the presence of a biosurfactant. In the case of no biosurfactant activity, the upper layer will turn cloudy but remain red. The assay is simple, and with the help of tools such as a Cybi-Disk robot, it can be applied in high-throughput screening [43].

2.1.6 Tilting Slide Test

This test examines the flow of water droplets over a glass slide surface and can be applied in preliminary screenings [44]. A single test colony picked from an agar plate is applied onto a sterilized glass slide near the glass edges and mixed with 1% saline water. The glass slide is gradually tilted to observe the flow of the water droplet over the glass surface, and activity is recorded if it flows. The method is easy to apply and does not require any expensive or specialized equipment [25], but serves only as a preliminary screening method and must be supported by secondary screening.

2.1.7 Victoria Pure Blue BO (VPBO) Assay

This assay is based on the surfactant-dependent solubilization of Victoria Pure Blue BO, a hydrophobic blue dye typically used in ballpoint pens. Assay plates are prepared before the screening by immobilizing the dye on 96-well polystyrene plates. The surfactants in aqueous solutions applied to the wells, e.g., supernatants of bacterial cultures can re-solubilize the dye into the liquid [16, 45]. The solubilized dye can be quantified, if desired, after the transfer of the liquid to a clean plate (Fig. 5) via the specific absorption at 625 nm. The method, initially used to determine residual detergent levels in medical preparations, offers a broad range of applications beyond the qualitative high-throughput screening for biosurfactant production, including biosurfactant quantification, e.g., for the comparative evaluation of different cultivation conditions and assessment of the CMC and solubilization properties of isolated surfactants [16, 46]. The VPBO Assay has been shown to be suitable for chemically different ionic and non-ionic biosurfactants; however, like

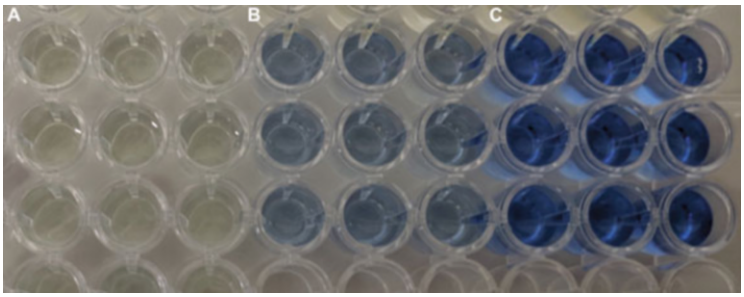

Fig. 5 Biosurfactant solutions after VPBO Assay in a microwell plate. The presence of surfactant is indicated by the blue dye Victoria Pure Blue BO that is released from the surface of the plate well by surfactant activity. This response (**b** + **c**) is concentration dependent, whereas in the absence of surface-active agents (or in amounts below CMC) the solution remains uncolored (**a**)

for the drop collapse assay, water solubility of the biosurfactant and a concentration above the CMC appear to be a prerequisite.

2.2 Targeted Screening Assays

The assays described here encompass two parts: methods that assess specific biochemical or physicochemical characteristics and performance of the biosurfactant; and those that directly measure surface and interfacial tension reduction.

2.2.1 Emulsification After 24 h (EC24)

The emulsification capacity index (EC24) measures the ability of an emulsifier to stabilize the emulsion of immiscible liquids over 24 h. After the mixing of two immiscible phases, for example, water and oil, the unstable emulsion divides into separate phases depending on the respective densities, whereas in the presence of a biosurfactant with emulsification capabilities, the emulsion is stabilized through interfacial tension reduction between the immiscible phases allowing them to readily mix (Fig. 6). This method, therefore, assesses the capability of a culture (or supernatant) to form an emulsion with a hydrocarbon such as paraffin, kerosene, and hexadecane after they are mixed [47]. The EC24 is measured as the height of the emulsification over the total height of the two-phased mixture, 24 h after mixing by agitation to form emulsions. Alternatively, emulsification can be detected by quantifying the turbidity from the emulsion using a turbidimeter [48].

However, the ability of a biosurfactant to form an emulsion is rarely associated with its surface and interfacial tension reduction potential [31, 37]. Therefore, good emulsion does not necessarily equate to surface and interfacial tension reduction [9],

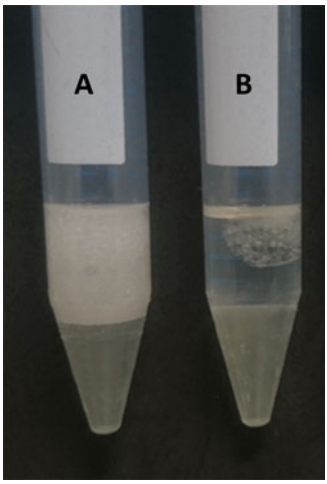

Fig. 6 Emulsification after 24 h, after mixing paraffin with culture supernatant. Following the mixing of two immiscible phases an unstable emulsion divides into separate phases (**b**), whereas a stabilized emulsion is formed due to the presence of recombinantly expressed lyso-ornithine lipid (LOL) in the cell-free culture media (**a**)

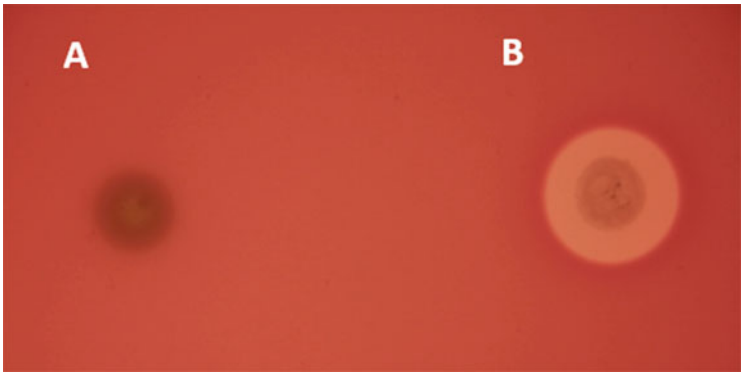

Fig. 7 The blood agar method detects hemolytic capabilities of some biosurfactants (**b**) through lysis of erythrocytes cells, producing a colorless transparent ring around the agar well, compared to no clear zone for a control solution (a)

and vice versa, biosurfactants capable of reducing the surface and interfacial tension are not necessarily good emulsifiers.

2.2.2 Hemolytic Assay

The blood agar method is widely used to detect biosurfactant production through the lysis of erythrocytes cells producing a colorless transparent ring around the colonies (Fig. 7) [49, 50]. It is a semi-quantitative method as the concentration of biosurfactant correlates to the linear increase of the diameter of lysis on the blood agar. The assay is also suitable for a 96-well and liquid format where the amount of hemoglobin released into the solution is determined photometrically [51]. However, hemolysis is not a universal property of biosurfactants. Furthermore, hemolysis may

also be evoked by pore-forming proteins or phospholipases [52] which can yield false-positive results. Hence, hits from this assay must be treated with care and maybe not as the method of choice for primary screenings.

2.2.3 Colorimetric Complex Release Assays

The complex formation of biosurfactants with cationic detergents, resulting in the displacement of a dye molecule, can be applied to determine the presence of biosurfactants in supernatants photometrically at 96 well scale.

CTAB-Methylene Blue Plate Assay

The cetyltrimethylammonium bromide (CTAB)-methylene blue method, also referred to as the "blue agar plate" method, serves as a semi-quantitative assay for the preliminary detection of extracellular anionic surfactants [17]. The positive detection of biosurfactants on agar containing 0.5 mg/ml CTAB and 0.2 mg/ml methylene blue is seen by the formation of a blue halo surrounding a bacterial colony or sample (Fig. 8). The blue halo is formed through the binding and forming of a complex of anionic surfactant with the cationic surfactant of CTAB. Not all bacteria can be screened using the method because CTAB is toxic to some bacteria including *E. coli* [31].

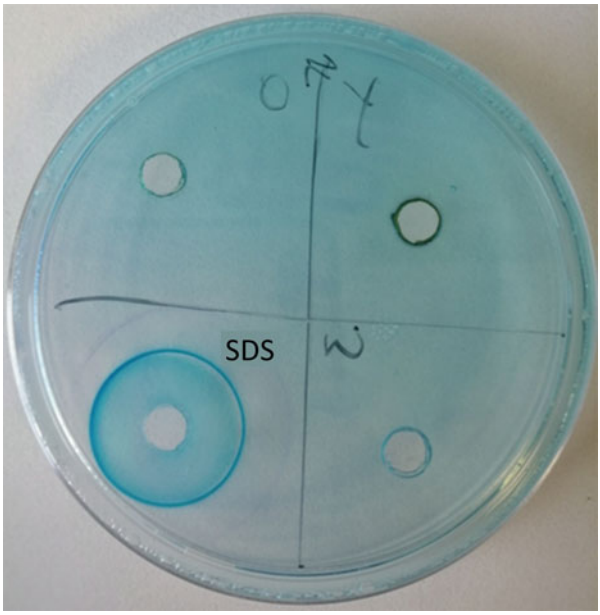

Fig. 8 The CTAB-methylene blue method for the detection of extracellular glycolipids or other anionic surfactants. SDS shows the positive detection of surfactants as seen by the formation of a blue halo

CPC-Bromothymol Blue Assay

Cetylpyridinium chloride (CPC) in combination with bromothymol blue (BTB) or fluorescein has been successfully applied in a similar manner as the CTAB-methylene blue assay [18, 53]. Biosurfactant presence and concentration can be determined via the color/fluorescence shift evoked by the displacement of the dye components. This strategy was described as a very reliable method to even quantify the anionic lipopeptide surfactin [18] but it may interfere with media components or primary metabolites [54] and may therefore require extraction procedures before the assay. Furthermore, it is most likely that both complex release assays are restricted to anionic biosurfactants.

2.2.4 Detection of Biosurfactant Production by Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is mainly applied in the characterization of the chemical nature of the produced biosurfactants by using selective reagents and manipulating the polarity of solvents when separating crude extracts on a silica gel plate. For the detection of functional groups of biosurfactants, different staining reagents can be used, e.g. ninhydrin stains lipopeptides red whereas α-naphthol stains glycolipids purple (Fig. 9). Hydrophobic moieties like aromatic ring systems or lipid chains can be visualized applying iodine vapor or primuline. This method is not suitable for high-throughput screening.

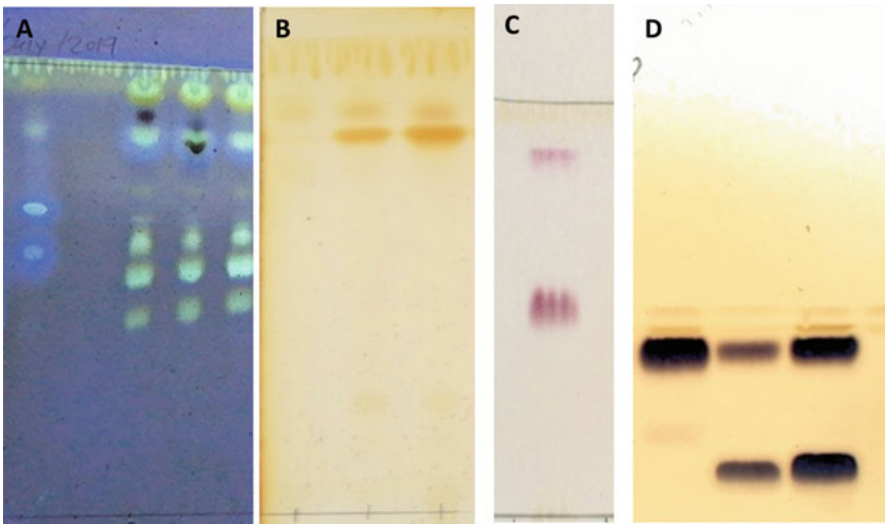

Fig. 9 TLC analyses of different biosurfactant extracts, (**a**) stained with primuline for lipid detection; (**b**) stained with iodine for lipid detection; (**c**) stained with α-naphthol for sugar detection; (**d**) stained with orcinol/sulfuric acid for sugar detection

2.2.5 Screening Methods Based on Cell Surface Hydrophobicity

Several indirect methods are suitable to screen for differences in cell surface hydrophobicity. The cell-bound biosurfactant production by a microorganism is associated with high hydrocarbon uptake and therefore high surface hydrophobicity, whereas microorganisms that release biosurfactant extracellularly are associated with low surface hydrophobicity. Many other factors influence the hydrophobicity of bacteria, such as physiological aspects like growth conditions and cellular age. Therefore, these methods are generally used for rapid identification during the isolation stages and are followed by secondary screening.

Hydrocarbon Overlay Agar

Microbial isolations on oil-coated agar plates can identify strains that degrade hydrocarbons and produce biosurfactants by the production of an emulsified halo surrounding the colony [36, 55]. The method is easy to set up and does not require special equipment, but it cannot be applied for strains that do not degrade hydrocarbons. The method is used for initial screening purposes only and requires further confirmation of positive results.

Bacterial Adhesion to Hydrocarbon Test (BATH)

The photometrical bacterial adhesion to hydrocarbons (BATH) assay measures the hydrophobicity of a cell surface by measuring the degree of adhesion of washed microbial cells to different hydrocarbon compounds, such as hexadecane or octane [56]. Hydrophobicity of the cells is measured by assessing changes in absorbance at 550 nm of the lower aqueous phase before and after the mixing procedure, expressed as a percentage. The basic principle is that a decrease in the turbidity of the aqueous phase correlates to the hydrophobicity of the cell; however, it is considered one of the least reliable methods [30].

Hydrophobic Interaction Chromatography (HIC)

This chromatography screening method is based on the hydrophobic interaction between non-polar group regions of particles and the non-polar groups on a hydrophobic chromatographic resin. It was initially employed to purify and separate biomolecules based on differences in their surface hydrophobicity [27] and was modified for biosurfactant screening. A bacterial suspension in a high-salt buffer is allowed to flow through a gel bed of hydrophobized Sepharose to which hydrophobic cells adhere. Turbidity and bacterial counting in the elute are measured to obtain the degree of adsorption of the cells to the gel. Stepwise or continuous decrease of

the ionic strength for desorption of the adherent microbes promotes their elution. This way, microbes of different surface hydrophobicity can be separated. The main advantage of applying HIC for screening biosurfactant production is that it is convenient because both isolation and screening of strains can be achieved simultaneously while also serving as a good comparative analysis of the hydrophobic properties of microorganisms.

Replica Plate Test Assay: Adhesion of Bacteria to Hydrophobic Polystyrene

This simple assay is used to identify and isolate hydrophobic microorganisms by their adhesion to hydrophobic polystyrene by pressing a flat sterile disk of polystyrene onto agar containing the colonies [57]. The underlying principle is that the affinity of bacteria to polystyrene strongly correlates to the hydrophobicity of the cell surface [58]. The advantage of this method is that hydrophobic strains can be simultaneously isolated and identified.

Salts Aggregation Assay

This assay involves the precipitation of cells by increasing salt concentration, the same principle used in salting out proteins [59]. The underlining principle is that the more hydrophobic the surface of the cell, the lower the salt concentration that is required to aggregate the cells. A bacterial suspension is mixed with various ammonium sulfate concentrations on glass depression slides and monitored for the formation of a white aggregate.

Solubilization of Crystalline Anthracene

This quantitative assay is based on the solubilization of anthracene, a highly hydrophobic crystalline compound, when added to the culture supernatant [60]. The production of biosurfactant is determined by measuring the concentration of the solubilized anthracene at 354 nm with a photometric device. Important to note is that the cell-free supernatant of the culture is used to conduct the assay, since bacteria could metabolize the anthracene, which may be mistakenly interpreted as biosurfactant activity [61, 62].

2.2.6 Structure-Based Screening as a Recent Advance in Physicochemical Screening Methods

The typically low biosurfactant titers in broth cultures not only impact the efficiency of the screening process, but also impede the structural determination of the isolated biosurfactants which relies on a large amount of purified compound and

time-consuming methods. To overcome these bottlenecks, Sato and co-authors [23] recently demonstrated a structure-based screening method in the early stages of the screening process. The approach they developed relies on the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS), a technique that is commonly used to identify organic molecules based on precise mass-to-charge ratio measurements. Although MALDI-TOF/MS has been applied for the structural determination of purified biosurfactants [63–67], this study has been the first to employ it for screening for biosurfactant producers and on crude samples. Currently, this method has only been applied for the screening of glycolipid-type producers, and therefore its application to other classes of biosurfactants has yet to be ascertained. Since the technique is premised on identifying mass to charge ratios of known compounds, its ability to identify completely novel structures is most likely very limited. However, this does represent a rapid and reliable tool to detect variants of a class of compound, as was demonstrated by Sato et al. [23]. Additional advantages of this approach are that MALDI-TOF/MS requires a small amount of sample for measurement and can distinguish structural differences in mixtures because of the precise measurement of the mass-to-charge ratio corresponding to the molecular weights of the compounds. Furthermore, it can be used to screen unidentified microbial cultures in addition to new biosurfactant variants. The fact that the structure can already be ascertained in the primary screening of the crude extract is an obvious advantage for accelerating the screening process.

2.2.7 Quantitative Screening Methods Based on the Direct Measure of the Surface and Interfacial Tension

Surface and interfacial tension reduction is the common measure of the physical property of surfactants, defined in two ways; the tension force per unit length exerted by a liquid in all directions at an interface of a solid or another liquid; or, explained in terms of energy, as the amount required to decrease the interior forces of bulk liquid molecules and molecules at the interface of the liquid in contact with other surfaces [68, 69]. The surface tension is measured as dimensions force/length in the units dyne/cm or mN/m. At a value of 72 mN/m, water is known to be one of the organic liquids with the highest surface tension value [10]. An effective surfactant should interrupt the forces per unit length of water and lower the surface tension from 72 to 30 mN/m [9]. Interfacial tension (IFT) is the intermolecular attractive force of the molecule in a liquid and represents the emulsion capacity of a surfactant. A high emulsion relates to a low IFT [10]. A surface-active biomolecule must lower the interfacial tension for water against *n*-hexadecane from 40 to 1mN/m.

Tensiometers can be universally used to quantify the activity of any biosurfactant by measuring the change in surface and interfacial tension at their air/water and oil/water interfaces. Force or optical tensiometry are techniques that are commonly used to measure the surface and interfacial tension. Force tensiometry involves a direct measure of the surface or interfacial force exerted on a probe, whereas the

optical tensiometry unit measure is calculated from theoretical equations whereby an image profile of a drop or bubble is extracted and fitted to an equation.

The reduction of the interfacial tension in air/water or oil/water systems increases with (bio)surfactant concentration but only up to a critical point, above which no further surface tension reduction occurs, instead, surfactants aggregate to form structures like micelles, bilayer, and vesicles. The value at the critical point is known as the critical micelle concentration (CMC) [9]. The efficiency of the surfactant is determined by how low its critical micelle concentration is, the lower the CMC, the less product needed to reduce the surface and interfacial tension. In most industrial processes, ranging from drug delivery systems to agricultural remediation technologies, the CMC value is considered particularly important for determining the biosurfactants' suitability in the respective application [70]. Discussed below are some of the direct screening techniques:

Du-Nouy-Ring Method

This is a traditional technique used for the direct measurement of surface or interface tension change generally using an automated tensiometer [36]. The method involves measuring the force required to pull through a fully submerged ring or loop of platinum wire from an interface or surface of the liquid of interest. The detachment force is measured relative to the surface or interfacial tension. A biosurfactant-containing solution is noted as one that reduces the tension of pure water to 40 mN/m or less [68, 69]. This measure shows a direct relationship to the drop collapse, oil spreading, and surface tension assays. It is the most widely applied method as it is accurate and easy to use; however, it requires specialized equipment [71].

Wilhelmy Plate Method

This is a universal method for measuring surface or interfacial tension at an air–water or water–water interface. A thin Wilhelmy plate is submerged perpendicular to the air–liquid or liquid–liquid interface and the force applied on it is measured [72]. The Wilhelmy plate is often made from filter paper, glass, or platinum with a rough surface to ensure wetting. The choice of material is not particularly important, but the material must have the capacity to be wetted by a liquid. The advantages of this method include that one can use disposable papers; it is considered the simplest and most accurate measure; it does not require correction factors when calculating surface tension, because the Wilhelmy plate assumes a zero-contact angle with the liquid; and there is no need to measure or know the density of liquids, only mass of plate and wetting force is considered [36]. The disadvantage of the application is that a large volume of liquid is needed.

Stalagmometric Method

This is one of the most common methods adopted to screen for biosurfactant production, using a traube stalagmometer to measure surface tension activity [73]. A pipette with a broad flattened tip is used as the capillary system, with which large drops of reproducible size are suspended from the tip, drop once a maximum weight/volume is reached. The volume is calibrated by the stalagmometer and the weight of the drop is dependent on the characteristics of the liquid being tested. The underlying principle is that the weight of volume is in equilibrium with the surface tension. Biosurfactant production is measured by counting the number of each drop that falls per volume from the glass capillary tube, and by measuring the density of the sample and the surface tension of the control sample liquid used, which is normally water. The main disadvantage of this screening method is the large variability usually obtained in the results, suggested being due to drop formation being too fast and not allowing the complete adsorption of the surfactant to the newly generated drop surface [10]. Another disadvantage is that consecutive measurements are not possible.

Axisymmetric Drop Shape Analysis by Profile (ADSA-P)

This optical method is used to simultaneously measure liquid surface tension and contact angle from the profile of a droplet resting on a solid surface [74]. The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension; biosurfactant solutions with low surface tension tend to minimize the surface area of the drop causing the droplet to deviate from a perfectly spherical shape when compared to those with high surface tension, as indicated by the drop collapse test (Sect. 2.1.1). The circumference of a liquid on a solid surface is captured as an image and the measurements are subsequently fitted to the capillary Laplace equation to calculate the surface tension [68, 69, 75]. The advantage of the ADSA-P is the small volume needed for the drop shape analysis. However, the shortcomings of using this method include the requirement for a camera and software; and it involves complex calculations; complex computational routine and samples cannot be measured in parallel [10, 36, 75].

Pendant Drop Shape Technique

This is the most common optical tensiometry method used to screen for biosurfactant activity and is considered an excellent screening technique for a quick analysis of surface and interfacial tension and measurement of contact angle [74]. The surface and interfacial tension properties are measured from a drop of liquid allowed to hang from the end of a capillary. The drop adopts an equilibrium profile based on the tube radius, the interfacial tension, its density, and the gravitational field [76].

3 In Silico Screening of Sequence Datasets for Novel Biosurfactants

The development and continuous improvement of next-generation sequencing (NGS) platforms and the subsequent scalability, cost reductions, and development of in silico tools have enabled the advancement of sequence-based screening for the discovery of novel biomolecules. The availability of genome sequences for over 800,000 bacteria and 4,000 fungi [77], as well as enormous metagenomic sequence datasets in public databases, representing largely untapped resources from diverse organisms from almost every environment imaginable, makes sequence-based screening particularly attractive. Not only can researchers bypass the sampling and isolation of strains and/or (meta)genomic DNA and sequencing costs, it enables also the convenient de novo synthesis of the identified genes and pathways, cloned into a plasmid vector of choice, through a number of service providers [78]. Without question DNA sequencing technologies have reinvigorated the discovery of new microbial enzymes and secondary metabolites [79, 80], but it has had rather limited application for the discovery of novel biosurfactants. However, several genome-guided efforts involving biosurfactant-producing strains have reinforced the notion that targeted sequence-based approaches have the potential to contribute to biosurfactant discovery. The following sections will outline the sequence-based screening process and discuss the potential for biosurfactant discovery.

3.1 Gene/Pathway Identification

Assembled genomes and/or contigs are queried to identify protein-coding sequences based on homology to reference sequence data in curated sequence databases where open reading frames (ORFs) or conserved protein motifs (for example active sites/domains) are identified through similarity search algorithms (e.g., BLAST [81], COG [82], KEGG [83, 84]. In many cases, expression of secondary metabolites, which encompass biosurfactants, involves more than one gene for biosynthesis. One of the most popular tools for the identification of biosynthetic gene clusters (BGCs) in general is antiSMASH (antibiotic and secondary metabolite analysis shell) [85]. This tool is continuously developed and currently provides researchers with an easy-to-use, up-to-date collection of state-of-the-art annotated BGC data. It identifies all BGCs present in the query sequence as well as facilitating cross-genome analyses. In addition to cluster predictions, more complex searches can be implemented via the graphical query builder from which researchers can gauge the novelty of the clusters, and by extension, the novelty of the compound they encode. Therefore, in addition to discovery, sequence-based mining can also serve as a dereplication tool that can be used to prioritize strains most likely to produce a novel biosurfactant. Advantageously, sequence-based screenings can be conducted in an ultra-high-throughput manner provided suitable bioinformatics and

computational capacity is available. Another advantage of sequence-based mining pertains to the realization that there is great disparity between the number of putative secondary metabolite gene clusters identified based on bioinformatics analyses vs the actual number of secondary metabolites detected from culture fermentations [86]. Such BGCs are regarded as cryptic, where either the genes are not expressed under standard laboratory cultivation conditions or they encode compounds that are produced in yields insufficient for direct isolation and characterization [80]. This not only points to an astounding level of as yet undiscovered metabolites for the majority of cultured organisms, but it also provides the opportunity to employ genome-guided strategies to identify biosurfactant BGCs in organisms that may have initially tested negative in a functional screen [87].

Given the structural and biosynthetic diversity of biosurfactants, there is no universal sequence-based screen to detect biosurfactant genes or pathways from datasets a priori [31]. For example, glycolipid discovery using a sequence-based approach is currently not viable due to the lack of a specific conserved domain to distinguish glycolipid associated enzymes from the wealth of glycosyltransferases performing a multitude of functions in bacteria [88, 89]. In consequence, glycolipid operon prediction is not implemented e.g., in antiSMASH. However, for lipopeptide biosurfactants which are encoded by nonribosomal synthase (NRPS) gene clusters, the conserved adenylation domain of the synthase gene could be targeted to identify NRPS BGCs, followed by a secondary screen of the initiation domains to discern for lipopeptide-specific NRPS BGCs [31]. For example, holrhizin A, a novel linear lipopeptide from the *Burkholderia rhizoxinica,* an endosymbiont of the rice seedling blight fungus *Rhizopus microspores,* was discovered through such a genome mining approach [90]. The identification of a conserved cryptic NRPS gene cluster among all sequenced Rhizopus endosymbionts led to the isolation and characterization of holrhizin A as a biosurfactant. It was further demonstrated to influence the formation of mature biofilms and thus cell motility behavior, typical for biosurfactants, and thus ultimately supports the colonization and invasion of the fungal host, furthering the understanding of the mechanism behind the exceptional *Burkholderia-Rhizopus* symbiosis relationship.

The discovery of holrhizin A represents an elegant example of how a genomics-led discovery not only resulted in the description of a novel biosurfactant, but also provided a functional link between orphan NRPS genes and a chemical mediator that promotes bacterial invasion into the fungal host. But this example also serves to highlight that sequence alone could not have predicted the biosurfactant properties of the NRPS-encoded metabolite. Therefore, it can be reasonably expected that a purely sequence-based screening approach will have limited success for biosurfactant discovery. A much higher success rate can, however, be expected when employing sequence-based screening for strains with confirmed biosurfactant activity, especially in the case where the primary or secondary screening already points to a specific class of biosurfactant which can inform the sequence-based search [91–93].

Another limitation of sequence-based screening pertains to the level of novelty that can be identified. Since sequence-based searches rely on similarity algorithms that score based on sequence identity, the discovery of completely novel

biosurfactants will be limited; and true novelty may be overlooked as very distantly related sequences are often unlikely to be found by homology-based searches [94]. However, a sequence-based approach could have enormous value in identifying variants of certain classes of biosurfactants. For example, rhamnolipids encompass a wide diversity of congeners and homologues [95], the synthesis of which are encoded by the *rhlA*, *rhlB,* and *rhlC* genes. Perhaps, sequence variations in the genes could be a proxy for predicting novel homologues and therefore sequence-based screening could identify new targets to validate by culturing the native host or through heterologous expression of the genes (discussed further below). Especially the products of BGCs that are not readily expressed by the natural producers under the chosen growth parameters may be accessed using heterologous expression and/or genetic engineering approaches to bypass the strict regulation systems in the natural hosts [96].

3.2 *Heterologous Expression of Putative Biosurfactant-Encoding Genes/Pathways Identified Through In Silico Mining*

Once identified, the genes or pathways can be cloned and heterologously expressed in a suitable host, to produce the biosurfactants for purification and characterization. The transfer of complete biosynthetic pathways is a considerable challenge because the respective genes may be dispersed over the chromosome (as for example the genes necessary for di-rhamnolipid biosynthesis in *P. aeruginosa*) [68, 69] or organized in very large BGCs. For the NRPS-synthesized lipopeptides, where BGCs can span >50 kb in size, standard cloning procedures involving polymerase chain reaction (PCR) amplification are not suitable and de novo synthesis approaches would not be feasible [97, 98]. However, if identified from genomes of culturable organisms, a large collection of tools has become available for generating conventional genome libraries and/or capturing even large BGCs in clones which can then be screened (in the case of a genome library) or assessed for biosurfactant activity [5, 99].

Even if complete BGCs are transferred to suitable host strains, the successful expression and biosynthesis for a specific biosurfactant is to a large extent dependent on the chosen host strain that must be suitable to produce a respective biosurfactant in amounts at least sufficient for detection and structural elucidation. This typically requires promoter recognition (or a suitable promoter sequence on the applied vector backbone), efficient translation of the foreign genes, supply of accessory proteins and cofactors, supply of precursor metabolites, and tolerance toward the surface-active and often also bioactive product itself. For example, Gram-positive bacteria like *Bacillus* or *Staphylococcus* are very susceptible to surface-active compounds in general, most likely because of the lack of a protective LPS-containing outer membrane [100]. Hence, although Gram-positive organisms are often pronounced

lipopeptide producers, they appear to not be favorable hosts to produce recombinant biosurfactants at high levels. The dependence of active production of NRPS machinery on a phosphopantetheinyl transferase (PPT) for posttranslational modification exemplifies the importance of suitable enzymatic capabilities of a host strain [101]. PPTs are often not part of BGCs and therefore must be supplied by the host in these cases, preferably with a broad substrate spectrum. Another consideration for host selection is for the host strain to lack biosurfactant/bioemulsification activity itself, to facilitate the isolation and characterization of the recombinantly expressed biosurfactant.

Approaches toward recombinant biosurfactant production are so far mainly focused on different proteobacteria like *E. coli* [102, 103], *Burkholderia* sp., and several non-pathogenic members of the *Pseudomonas* genus [103] as host organisms. In this context, the well-explored production of rhamnolipids in *P. putida* exemplifies the potential of recombinant production in a strain that combines high tolerance, a versatile metabolism, sufficient precursor supply, and low background activity with effective expression to enable high-yield production and, besides that, tailoring of biosynthetic pathways [104, 105]. With respect to recombinant lipopeptide production, *Bacillus* sp. and *Streptomyces* sp. as naturally potent lipopeptide producers have been additionally applied as hosts [5].

Recombinant production strategies have been largely based on prokaryotic host organisms so far. However, fungi represent a technologically important class of biosurfactant producers well-known for the production of sophorolipids, mannosylerythritol lipids, cellobiose lipids or hydrophobines and functional expression of related BGCs of fungal origin can often not be achieved in prokaryotic hosts. To this respect, there is a need to complement the established set of microorganisms for recombinant biosurfactant production with eukaryotic expression systems. Accordingly, a few initial studies report successful functional heterologous expression of (partial) biosynthetic pathways for biosurfactants in *Saccharomyces cerevisiae*, *Starmerella bombicola,* or *Pichia pastoris* [84, 106–108].

4 Metagenomic Biodiscovery: Unlocking Hidden Diversity

It is acknowledged that diverse and complex microbial communities inhabiting many unique niches remain undiscovered; yet could represent the source of enormous biosurfactant novelty. Moreover, researchers have for decades appreciated the difficulty of bringing the abundant microbial diversity to culture in the laboratory. To overcome the culturing limitations and to explore the natural wealth beyond the minority of microorganisms that is culturable, metagenomic screening approaches have been established in the hope of accelerating biodiscovery, and to specifically tap into novel chemical space [96, 109]. Metagenomic approaches for the identification of novel biomolecules utilize both approaches that have been explained in the previous sections, functional, activity-based screenings of metagenomic libraries constructed in a heterologous host; and sequence-based screening via bioinformatic

analysis of next-generation sequencing data of environmental DNA (eDNA) [21, 84]. For reasons that have already been eluded to, functional metagenomics (as opposed to sequence-based) has been considered the more promising approach for the discovery of novel biosurfactants [31, 84, 110]. However, only few studies have so far employed metagenomic screening successfully for surface-active metabolite discovery, in which novel genes encoding the synthesis of N-acylated amino acids are largely reported. N-acyl amino acid synthases (Nas) catalyzing the synthesis of acylated aromatic amino acids appeared rather frequently in functional screens for active compounds against Gram-positive bacteria [111]. However, aromatic N-acylated amino acids show pronounced surface activity and were, hence, detected in functional screenings for biosurfactants [112]. Considering the susceptibility of Firmicutes toward surfactants, it would be reasonable to assume that the observed antibiotic effect of many biosurfactants is connected to their surface activity. A recent study showed the advantage of different expression hosts even for such simple molecules by indicating that the expression of the same *nas* gene in different Proteobacteria led to different products [113]. Whereas *E. coli* extracts contained predominantly N-acyl tyrosine, *P. putida* produced mainly N-acyl-phenylalanine. In *P. koreensis* extracts, N-acyl-leucine was detected additionally.

Recent functional screens for surface activity revealed a structurally different biosurfactant from the family of acylated amino acids, namely lactamized lyso-ornithine lipids and ornithine lipids [40]. Key to success in this study was the simultaneous screening in different hosts, because the respective library clone showed surface activity only in the *P. putida* screening, whereas it was not detectable in the *E. coli* library, although later experiments proofed the functionality of the responsible metagenomic acyl-ACP-ornithine acyl transferase in *E. coli.* Here again, the different strains produced biosurfactants of different composition, with only *P. putida* producing ornithine lipids. Interestingly, a chromosomal integration shuttle vector was applied here for screening in *P. putida*, which might be a useful strategy to stabilize large eDNA fragments in the host cells.

The potential of especially functional metagenomics for the detection of completely novel biosurfactants was recently illustrated by the discovery of MBSP1, a protein with emulsifying properties [114]. Remarkably, this protein is putatively of archaeal origin whereas the known classes of emulsifying proteins, hydrophobins and cerato-platanines, are produced by fungi [115, 116]. Hence, MBSP1 represents the first example of a prokaryotic homologue. Moreover, sequence searches revealed that homologues of MBSP1 are common among *Halobacteriaceae* but were previously unassigned to a function. Although advances are being made in computational tools to improve predictions of hypothetical proteins which represent a significant fraction of the sequences in public databases [117], this study elegantly exemplifies functional metagenomics as the most reliable method for determining the functionality of novel protein sequences, a task which is currently beyond the capabilities of existing in silico annotation technologies [94].

The available studies on functional metagenomic identification of biosurfactants commonly reported surface-active molecules retractable to the activity of one protein, whereas the biosynthesis of the majority of known biosurfactants is encoded

in operons of several genes or even in large BGCs. This bias may, in part, be related to the challenges associated with maintaining a complete biosynthetic pathway during library construction. In particular, with regard to lipopeptides, it is unlikely to achieve clones encoding a complete NRPS gene cluster in a plasmid, cosmid, or fosmid library with typical average fragment sizes of 5–40 kb [97, 98]. Although there are examples for very compact NRPS machineries (e.g., Serrawettin W1 synthesized in an iterative mode by only one NRPS module [118]), to successfully access this class of biosurfactant from uncultured representatives, metagenomic libraries need to ensure fragment sizes greater than 40 kb. This not only presents an enormous challenge to the handling and maintenance of DNA fragments of this size from environmental samples, there are also very few vectors and expression hosts available for the construction of such libraries [31, 119]. Alternatively, lipopeptide gene clusters can be reassembled from sequence-based screenings and cloned for recombinant expression as discussed earlier; however, metagenomic sequencing datasets are overly complex and prediction and correct reassembly of gene clusters from several contigs remain a challenge [120, 121].

Occasional reports on the discovery of novel lipopeptides through metagenomic approaches illustrate that sequence-based approaches targeting this group may be feasible despite the mentioned challenges. Nonribosomal peptides in general are well-known for their bioactivities, e.g., as antibiotics or anticancer agents. Hence, many studies were conducted with this focus to identify novel nonribosomal peptides and the related biosynthetic machinery using sequence-based screening approaches to explore novel pharmaceutic lead structures [122]. Several of those studies revealed lipopeptides which were, however, not tested for surface activity yet most likely exhibit it because of the amphiphilic structure of the molecules. Recently discovered examples are the antibiotics cadaside A and malacidin A with massively charged cyclic peptide headgroups [123, 124]. Here, the adenylation modules incorporating the charged amino acids were used as target for sequence-based screenings for novel calcium-dependent antibiotics. Another likely biosurfactant candidate is humimycin A, a linear lipopeptide with activity against *Staphylococcus* that was resynthesized according to a NRPS machinery architecture discovered in metagenomic sequence data [125].

Strikingly, most of the successful recombinant biosurfactant production examples described utilize biosynthetic genes from related strains, e.g., *P. aeruginosa* rhamnolipid genes were used to establish rhamnolipid production in *P. putida*. Such a strategy appears advantageous to avoid issues of promoter/RBS recognition or differing codon usage. In this light, it appears as a strong limitation that *E. coli* is still overwhelmingly applied as host strain for functional metagenomic library screens, probably because well-established protocols and commercial kits for library construction are based on *E. coli* vectors. A number of studies reported accordingly the successful application of shuttle vectors to enable screening for natural product formation in different hosts, specifically in a range of proteobacteria including Pseudomonads and in actinomycetes [97]. Their results illustrated the huge advantage of considering screening strains other than *E. coli* or even parallel screening in different strains [31, 122, 126]. Different hosts did not only vary in their capabilities

to realize a specific biosynthetic pathway encoded by the introduced DNA, even the compounds produced from the same BGC differed sometimes in dependence of the host, as described before [40, 113, 127]. Furthermore, if the recombinant production of a certain biosurfactant is detrimental for the applied host, these novel biosurfactants will obviously remain undiscovered using only this specific host for the screening.

In this context the application of functional metagenomics using eukaryotic hosts remains comparably unexplored; probably because the reassembly of BGCs from intron-free metagenomic cDNA libraries, which are usually used to uncover single biocatalysts, appears challenging [128]. However, the bacteria-similar organization of biosurfactant-related genes in *S. bombicola* or *U. maydis* as a distinct gene cluster without or with just a few introns [129–131] suggests that yeast-based screening might be a useful tool to identify novel fungi-borne biosurfactants within classical eDNA libraries in the future.

5 Coming Full Circle: Culturing Considerations to Unlock Novel Biosurfactant Potential

Due to the "Great Plate Count Anomaly," referring to the orders of magnitude difference between the number of organisms that can be cultured on laboratory media vs. the numbers countable by microscopic examination [132], together with the availability of well-established culture-independent technologies with revolutionary impact, the merits of continued traditional culture-based approaches for the discovery of novel biosurfactants could be questioned. This is especially pertinent given the dearth in novel structures despite ongoing culture-based screening efforts, whereas metagenomics has more recently revealed novel biosurfactant classes [114]. Furthermore, genomics presents a number of advantages for overcoming the limitations associated with the culturing of microorganisms for biosurfactant discovery; both in terms of the inability to establish pure cultures of representatives of all bacterial divisions and accessing the number of biosynthetic pathways that remain "silent" in the native host. These will undoubtedly open new possibilities for biosurfactant discovery.

However, despite the genomic revolution, bringing a microorganism into culture is still essential for realizing its full potential [133], especially toward designing and operating stable and resilient high-performing systems required for industrial scale production. This is particularly relevant for biosurfactant discovery since in many cases the biosurfactant production is tightly linked to the physiology of the microorganism. For example, culture-based studies continue to bring new understanding of the various roles that biosurfactants play in quorum sensing and swarming motility and how this contributes to bacteria co-ordinating virulence and pathogenesis [134, 135]. The value of having the organism in culture to query experimentally provides unmatched opportunities to understand the microorganism in question

which may be crucial to the downstream aspects concerning the development of the biosurfactant for industrial application [136]. This highlights two major considerations for biosurfactant discovery:

1. Many biosurfactants will likely remain undiscovered if the producing microorganisms are not brought into culture. In other words, the success of discovering novel biosurfactants is as much dependent on the employment of novel isolation approaches as on the aspects concerning screening. Several advances have been made over the last decade to improve the culturability of rare and novel microorganisms and the reader is referred to a number of recent reviews on this topic [133, 136–140]. Although very much needed, not all currently proposed alternative isolation protocols are feasible with the screening technologies available; for example, where the isolation involves diffusion chambers due to a dependence on metabolic consortia [133].
2. Biosurfactant production may be strongly influenced by cell culture conditions and therefore the screening process needs to take cognizance of this. For example, the production of surfactants has been shown to be conditional on whether the bacteria are grown on a surface or cultured planktonically [141], while in others expression is influenced by growth stage [142, 143]. Therefore, the traditional approach to assess culture supernatants for biosurfactant activity may completely miss the discovery of those that are only produced when cultured on agar. While a move to more high-throughput screening methods is advocated as being key to the discovery of new biosurfactants [10], it needs to take into consideration the more tedious and slower One Strain Many Compounds (OSMAC) principles. OSMAC is a culture-based approach that involves the manipulation of easily accessible culturing conditions to induce the expression of all the biosynthetic pathways encoded by a single microorganism [144]. A major advantage provided by OSMAC is that it eliminates bias during biosurfactant screening because the stimuli responsible for the activation of the many biosurfactant pathways differ between microorganisms. This approach exploits the fact that microorganisms produce secondary metabolites as a defense mechanism against other organisms in nature [145] and that under stressful conditions, microorganisms tend to produce secondary metabolites either to adapt to the environment for self-defense or intercellular communication [146, 147]. Moreover, OSMAC is aimed at stimulating the expression of the "silent" biosynthetic pathways and should form an integral part of the screening process, to unlock expression of untapped microbial-derived biosurfactants [148]. Biotic and abiotic parameters such as nutrient content (carbon, phosphate, nitrogen sources, and trace elements), aeration levels and shape of the culturing flasks, physical parameters (i.e., pH, temperature, salinity, heat shock treatments), the effect of ethanol or organic compounds, the presence of precursors of secondary metabolites and co-culturing can be easily changed and may alter the global physiology of a microbial strain, and in turn significantly affect biosurfactant expression [80, 149]. It is important to note that the expression could be subject to regulation that might not be immediately linked to biosurfactant production and rather

associated with central physiological features and/or dependent on the coordinated expression of different pathways [80]. Furthermore, the triggers are organism and pathway specific [150], therefore systematic alterations to culture conditions need to be conducted. The empirical nature of the OSMAC approach is therefore time-consuming, laborious, and challenging from a practical standpoint, as the number of cultivation parameters that can be changed is virtually limitless. However, the rewards could be enormous as it offers the potential to further diversify the biosurfactant repertoire and improve the hit-rate to feed the biosurfactant pipeline, essential toward creating eco-friendly and cost-effective industrial scale production processes.

6 Concluding Remarks

There is a need and a gap to finding new biosurfactants with structural diversity suitable for specifically tailored applications in different industries beyond the three main products surfactin, sophorolipids, and rhamnolipids that are currently available commercially. In this chapter, we have highlighted the main areas that with concerted effort could help uncover much diversity that is undoubtedly still hidden in nature.

Firstly, increased effort in the application of a broad range of different screening methods has been highlighted as one of the solutions to finding new biosurfactant products. The screening methods over the years have been dominated by those mainly relying on the detection of surface or interfacial tension reduction and emulsion activity. One of the biggest challenges of using well-established assays is that it places a substantial restriction on the level of novelty that can be acquired, thus the same groups of biosurfactants will continue to be isolated. There has been little to no effort in developing alternative methods that assess properties other than the ones already screened for and therefore this is an area that calls for technological innovation to accelerate the discovery of novel biosurfactants. Sequence based screening has been presented in this chapter as an alternative approach to the more traditional activity-based screening of culture fermentations. Although the heterologous expression of biosurfactant pathways identified through in silico screening opens a number of opportunities for biosurfactant discovery and development, especially in the case of lipopeptide biosurfactants and for those encoded by "silent" BGCs, it remains to be seen the level to which novel chemistry can be revealed through homology dependent screening. The added advantages of employing an in silico screen followed by heterologous expression for biosurfactant discovery is that the cloning strategy can be designed to maximize the biosurfactant yield, and by using metabolically characterized hosts, the biosurfactant purification and characterization steps can be substantially accelerated in comparison with conducting this from the native host [151]. An important aspect to its success will be the ability to tackle some of the challenges associated with heterologous expression, most

notably, the development of a wider range of bacterial and fungal hosts and corresponding genetic tools to maximize the expression and production.

Secondly, the cultivation of microorganisms poses a significant limitation on biosurfactant discovery. On the one hand, metagenomic biodiscovery has the potential to contribute to biosurfactant discovery without the need to culture microorganisms, which holds much promise since only less than 1% of the microbial diversity is currently accessible through the traditional laboratory-based culturing [111]. Therefore, if this vast genetic repertoire is to be exploited and novel microbial-derived biosurfactants uncovered, it is clear that culture-independent approaches will need to be employed. On the other hand, it is recognized that the pure culture of microorganisms to gain insight into their physiological and cell-biological properties is essential to progressing biosurfactant discovery. The application of new isolation protocols has demonstrated that the number of species isolated can be doubled and allows the culturing of microorganisms corresponding to sequences previously not assigned [137], therefore we are far from having exhausted all culturing options. Even for already cultured isolates, extensive experimentation is needed to ensure that the right culturing conditions are used to trigger biosurfactant production, as there are potentially many undiscovered biosurfactants from previously isolated and screened microorganisms. Therefore, greater attention should be given to the isolation and culturing and not just the screening aspects of biosurfactant discovery, even though these may sometimes result in an overall long and costly route to undertake [80]. However, mature technologies such as micro-fermentation and experimental design represent promising strategies that in combination should streamline the discovery processes.

Finally, as with many other biodiscovery programs aimed to develop novel biocatalysts to improve industrial pipelines, the overall notion is that integrated strategies that include genomic and synthetic biology approaches have the potential to fast-track the discovery and subsequent improvement of a new generation of biosurfactants [152]. We have without question only scratched the tip of the iceberg with respect to biosurfactant diversity, and there is no single approach that will ensure that we fully realize all that Nature has to offer. Bottlenecks exist in both culture-based and culture-independent approaches; however, the continuous development of more efficient and powerful tools to explore the expansive potential harbored in natural environments will undoubtedly deliver novel biosurfactants.

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Parameters Influencing Lipase-Catalyzed Glycolipid Synthesis by (Trans-) Esterification Reaction

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Contents

Abstract Glycolipids are biodegradable, non-toxic surfactants with a wide range of applications. Enzymatic esterification or transesterification facilitated in reaction media of low water activity is a reaction strategy for the production of tailor-made glycolipids as a high structural diversity can be achieved. Organic solvents, ionic liquids, and deep eutectic solvents have been applied as reaction media. However, several challenges need to be addressed for efficient (trans-)esterification reactions, especially for the lipophilization of polar substrates. Therefore, crucial parameters in (trans-)esterification reactions in conventional and non-conventional media are discussed and compared in this review with a special focus on glycolipid synthesis.

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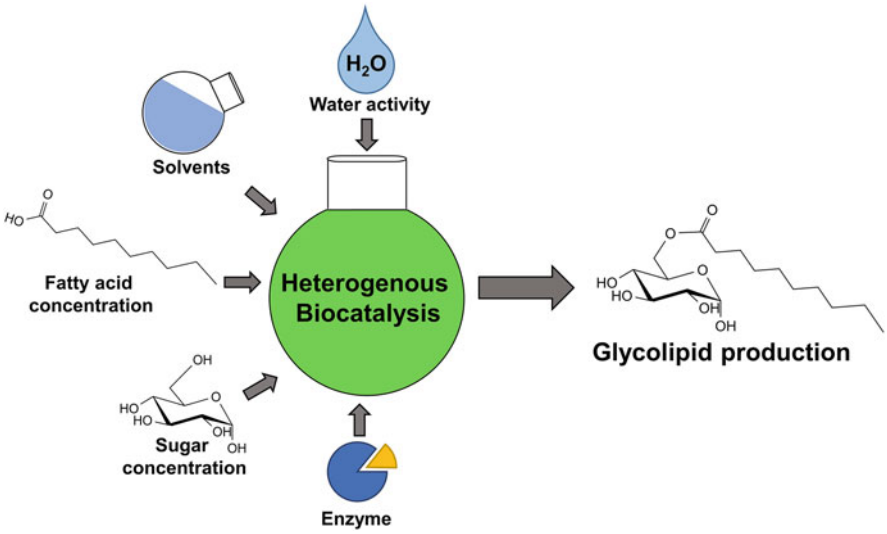
Graphical Abstract



Keywords Biosurfactants, Deep eutectic solvents, Enzymatic synthesis, Glycolipids, Lipases

1 Introduction

Glycolipids are non-ionic surfactants that are not of fossil origin and can be produced entirely based on renewables. They are more ecofriendly than petrochemically-derived surfactants as they pose no risk of accumulation in the environment because they are readily biodegradable [1–6]. Moreover, glycolipids are considered as non-toxic exhibiting no mutagenic potential, low toxicity toward invertebrate and zebra fish, as well as low cytotoxicity against human epidermal keratinocytes [3, 4, 6, 7].

Glycolipids were shown to have excellent surface properties: high surface activities in combination with an efficient lowering of surface tension [8–10]. They efficiently stabilize emulsions and foams [9, 11–13]. Therefore, they present a sustainable alternative to petrochemical surfactants.

Generally, surfactants have a wide field of applications in everyday life, as well as in industry. They are used in detergents, cosmetics and foods, as well as in fire-fighting and petrochemistry [14, 15]. Sucrose esters are glycolipids already approved for application in food industry [16]. Due to their drug permeability enhancing effects glycolipids are also of relevance for the pharmaceutical industry [17]. Moreover, antibacterial, anti-adhesive, antiviral, and tumor inhibiting activities are reported for glycolipids [10, 13, 18–20].

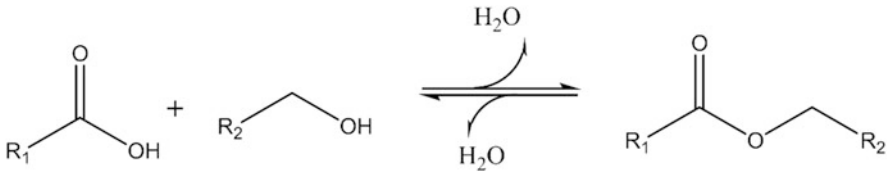


Fig. 1 Reaction scheme of reversed hydrolysis

Chemical synthesis, microbial fermentation, and enzymatic synthesis are possible strategies for glycolipid production. Chemical glycolipid synthesis is industrially established on a large scale by Fischer glycosylation, which ensures low cost production with high yields [21–23]. However, chemical synthesis also has a number of disadvantages: harsh reaction conditions are necessary using high temperatures and acidic catalysts [21–23]. Product mixtures are generated and products are formed which make a costly purification necessary [21–23].

Rhamnolipids, sophorolipids, and mannosylerythritol lipids are microbial lipids with commercial applications in cosmetic and detergent industry [14]. However, structural variety of glycolipids in microbial fermentation is limited to the metabolism of the host. Low glycolipid titers in fermentation broth render purification laborious and costly [24, 25].

Enzymatic synthesis is a method enabling the production of a nearly unlimited diversity of glycolipids [9, 11, 26–29]. Thus, the tailor-made production of glycolipids gets possible. Enzymatic synthesis is based on reverse hydrolysis, which can be catalyzed enzymatically under conditions of reduced water activity (Fig. 1). Hence, organic solvents, ionic liquids, and deep eutectic solvents (DES) are applicable reaction media [30–35]. The use of DES enables glycolipid production entirely based on renewables. A process solely based on lignocellulosic biomass was presented in 2018 by Siebenhaller et al. [36]. By application of a microwave reactor, even a one-pot synthesis of glycolipids from yeast biomass without previous extraction and transesterification of fatty acids was achieved [37].

This review discusses the latest findings on different parameters influencing enzymatic transesterification. Section 2 deals with deep eutectic solvents as they emerged only recently as green alternative to common solvents. Their properties and their health and environmental risk assessment will be addressed. Section 3 presents crucial parameters for enzymatic transesterification. Here, the role of different enzymes (Sect. 3.1), the impact of the sugar loading (Sect. 3.2), the influence of the fatty acid concentration (Sect. 3.3), and the role of water in the reaction systems (Sect. 3.4.) are discussed, as well as the impact of solvent nucleophilicity and solvent hydrophobicity (Sect. 3.5.).

2 Deep Eutectic Solvents

Deep eutectic solvents were first described in 2003 by Abbott et al. [38]. They are a mixture of two solid components, a hydrogen bond donor and a hydrogen bond acceptor, which result in a liquid at room temperature after heating or freeze-drying. DES are considered as supramolecular structures with hydrogen bond interactions [39–41]. A wide range of hydrogen bond donors and acceptors are applicable for DES formation which enable tailoring of the physicochemical properties of DES [42, 43]. There are hydrophilic, water-miscible DES and hydrophobic, water-immiscible DES, binary and ternary DES, as well as acidic, neutral, and alkaline DES covering a wide range of polarities [39, 44–49]. Due to this diversity, DES can be applied as "designer-solvents." DES have a high dissolution power, e.g. choline chloride: urea- and choline chloride: glycerol-DES, as well as ternary DES consisting of choline chloride or guanidine hydrochloride combined with ethylene glycol, propylene glycol or glycerol and p-toluenesulfonic acid are reported to dissolve up to 80% of xylan and lignin from biomass [50, 51]. DES are reported to have stabilizing effects on enzymes while their individual components lead to enzyme denaturation. Urea leads to denaturation and inactivation of *Candida antarctica* lipase B (CalB) by disrupting hydrogen bonds of the enzyme [52]. In choline chloride: urea- DES, diffusion of urea is limited due to the strong hydrogen bond network within the DES and the enzyme remains stable and active [52]. The DES forms hydrogen bonds with the surface of the enzyme resulting in a more rigid structure of the enzyme and an increased thermal stability [52]. In dissolutions of hydrophilic DES the supramolecular structure of DES is remained even with addition of up to 50% water, as water gets incorporated into the hydrogen bond network, only at higher dissolution the structure of DES gets disrupted [39, 41, 53].

In contrast to organic liquids DES are non-volatile and non-flammable [42, 43]. DES have some further advantages over ionic liquids: DES are easier to prepare than ILs and due to the low cost raw materials, DES cost only about 20% of ILs [54]. Furthermore, DES have a higher biodegradability and lower toxicity compared to ILs (see Sects. 2.1 and 2.2).

The applicability of DES-buffer mixtures for fed-batch and continuous processes was shown for the enzymatic esterification of glycerol and benzoic acid in 2019 [55]. Recently, also scalability of a DES system for glycolipid synthesis was proven [56].

2.1 Toxicity of DES

DES are less cytotoxic than ILs [57]. Choline chloride: amino acid DES show about 10 times lower inhibitory effects on enzymes than the imidazolium-based IL [Bmim][BF4] on acetyl choline esterase and the minimal inhibitory concentration toward catalase was even 600–800 times higher than those toward acetyl choline esterase

[58]. DES cytotoxicity is cell line dependent and depends on the hydrogen bond donor used [57]. DES with urea as hydrogen bond donor are less toxic than those with glycerol, ethylene glycol or triethylene glycol [57]. Interestingly, these DES show lower cytotoxicity than aqueous solutions of their single components which indicates a reduced reactivity after DES formation due the strong hydrogen bond network. Glucose based DES are less harmful than fructose based DES [59]. The sugars are metabolized differently in the cells which leads to a higher formation of reactive oxygen species in fructose metabolism compared to glucose metabolism [59]. The cytotoxic effects of DES are related to an increased cell membrane permeability and an increase in reactive oxygen species level [57, 59].

Toxicity of hydrophobic DES has still to be assessed more thoroughly. It is merely known that menthol: lauric acid DES exhibit cytotoxicity toward HACaT cells similar to pure menthol [60].

Choline chloride: amino acid DES also showed 10–200 times lower toxicity toward bacteria than imidazolium or pyridinium derived ILs [58]. DES based on choline chloride or choline acetate as hydrogen bond acceptors and acetamide, glycerol, ethylene glycol or urea as hydrogen bond donors exhibit low toxicity to bacteria at concentrations below 75 mM while they show antibacterial activity at high concentrations [61]. Inhibitory effects toward gram-negative bacteria were higher than toward gram-positive bacteria, suggesting a different mode of action than conventional bacteriocides, e.g. increasing cell permeability [58, 62].

Inhibitory effects of DES based on cholinium and alkanoates on growth of filamentous fungi decreased with increasing alkyl chain. The minimal inhibitory concentrations of all cholinium alkanoates were higher than those of SDS and benzalkonium chloride [63].

Choline chloride based DES show phytotoxic effects depending on the hydrogen bond donor, while the use of ethylene glycol and acetamide shows phytotoxic effects on garlic, urea- and glycerol-DES exhibited no significant phytotoxic effect on garlic [61].

Hydras are freshwater invertebrate used for ecotoxicological studies. Choline based DES exhibit lower toxicity on hydra than their single components and therefore also a lower ecotoxicological burden [61, 64].

2.2 Biodegradability of DES

Biodegradability of the solvents plays a major role in the evaluation of the environmental burden of manufacturing processes. Therefore, this is an important criterion in the selection of reaction media.

DES based on choline chloride with urea or acetamide are characterized as readily biodegradable while those with glycerol and ethylene glycol only showed biodegradability comparable to IL [61]. DES based on ChCl:amino acids were also readily biodegradable [58]. Likewise, the more hydrophobic DES consisting of cholinium hydrogen carbonate and fatty acids showed biodegradability [63]. In DES, a

correlation between low toxicity and high biodegradability was observed [58]. This simplifies solvent selection compared to ILs, since ILs of low toxicity usually show low biodegradability and therefore a high environmental burden [58]. However, there are only a few studies existing on the biodegradability of hydrophobic DES while these data are still missing for most hydrophobic, water-immiscible DES.

3 Enzymatic Synthesis

Success of biotransformations is strongly related to the choice of appropriate reaction conditions. Several parameters are already identified as crucial for enzymatic synthesis of glycolipids in organic solvents as well as in uncommon reaction media. Besides the selection of a suitable enzyme, the water content, substrate concentrations, and solvent properties such as nucleophilicity and hydrophobicity are decisive for efficient enzymatic synthesis (Table 1). These parameters will be discussed in detail in the following chapter.

Enzymatic glycolipid synthesis was demonstrated with three different enzyme classes: lipases, glycosidases, and proteases. Glycolipid production using proteases or glycosidases was less investigated than lipase-catalyzed synthesis.

Protease catalyzed synthesis of sugar fatty acid esters was successfully conducted in organic solvents using subtilisin and *Bacillus pseudofirmus* Al-89 protease [65–67]. 90% conversion was reached in a DMF/water-mixture using subtilisin [65] and 98% conversion to sucrose laurate in 9 h using Protex 6L protease in a tert-amyl alcohol/DMSO/water solvent mixture [67]. In a comparative study, Bernal et al. [68] reached 57% lactulose yield within 24 h using subtilisin and 61% using *Thermomyces lanuginosus* lipase in acetone [68]. So far, no studies on glycolipid

Table 1 Parameters positively influencing the efficiency of transesterification reactions

Parameter	Organic solvents	Ionic liquids	Deep eutectic solvents
Sugar loading	Supersaturated solution	Supersaturated solution	Supersaturated solution
Molar ratio of sugar and fatty acid	Equimolar	n.e.d.	n.e.d.
Water activity	$a_w < 0.2$	$a_w \sim 0.2$	$0.15 < a_w < 0.25$
Water content	Water removal system		Addition of water up to 10%
Solvent nucleophilicity	Low nucleophilicity	Low nucleophilicity	Low nucleophilicity
Solvent hydrophobicity		Medium polarity	
Others		Low halide content	

Table 1 shows which parameters were shown to work out most efficient for enzymatic glycolipid synthesis in the different solvent systems, using Novozym 435 as biocatalyst. *n.e.d.* not enough data for a clear evidence

synthesis using proteases in DES are available. Albeit, it was shown that subtilisin exhibits transesterification activity in choline chloride: urea DES [69].

Glycosidase catalyzed synthesis of glycolipids was conducted in organic solvents and biphasic systems [70, 71]. Miranda-Molina et al. [72] reported the first glycosidase catalyzed glycolipid synthesis in DES [72]. Organic acid containing DES inactivated α-amylase within 4 h while hydrolytic activity was still measureable after 4 h in choline chloride: urea, propanediol: choline chloride: water, choline chloride: glucose: water, and choline chloride: sucrose: water DES. However, at least 20% of the cosolvent water was necessary to maintain alcoholysis activity of α-amylase, in choline:chloride: glucose: water even 60% water was mandatory. At high DES concentrations reaction rates of hydrolysis and alcoholysis reaction were decreased with hydrolysis being affected more strongly. Selectivity of methyl-glucoside synthesis was higher in DES containing reaction media than in pure buffer [72]. Therefore, DES has potential for further investigations as solvent for glucosidase catalyzed glycolipid synthesis.

First lipase-catalyzed lipophilization of polar substrates in DES was reported 2013 by Durand et al. [73]. Water activity, solvent hydrophobicity, and solvent nucleophilicity are parameters that have already been identified as crucial for enzymatic glycolipid synthesis using lipases (Table 1).

3.1 Different Lipases for Transesterification

Several lipases have been screened for activity in DES. Novozym 435 revealed to be the most effective lipase for biodiesel production in DES, followed by Lipozyme TLIM while lipases from *Penicillium expansum*, *Aspergillus niger*, *Aspergillus oryzae,* and *Rhizopus chinensis* showed no or only little activity [64]. The study of Zhao et al. [74] demonstrated that the transesterification activity of Novozym 435 in DES is also higher than that of Amano lipase, porcine pancreas lipase, *Pseudomonas cepacia* lipase, and *Candida cylindracea* lipase in DES [74]. Novozym 435 also proved to be a more active enzyme in the synthesis of trehalose diesters compared to Lipozyme TLIM, porcine pancreas lipase, and *Carica papaya* lipase [12]. Moreover, Novozym 435 was the most effective lipase in sorbitol laurate synthesis in a 2-in-1-DES system consisting of sorbitol and choline chloride [56].

In a two-phase system of an IL and t-butanol Novozym 435 was the most active enzyme for glucose laurate synthesis with a conversion of 59%, while *T. lanuginosa* lipase reached 33% and *R. miehei* 8% [32]. *Pseudomonas cepacia* lipase, *Aspergillus sp.* acylase, *Candida antarctica* lipase A, *Candida rugosa* lipase were also tested in that system, but showed conversions of less than 5% [32].

In organic solvents Novozym 435 was also revealed as efficient biocatalyst. Novozym 435 showed superior performance in glycolipid synthesis in several studies compared to Lipozyme IM, *Candida antarctica* lipase A, and lipases from *Rhizomucor miehei*, *Thermomyces lanuginosa*, *Pseudomonas cepacia,* and *Fusarium solani* [35, 75, 76].

Novozym 435 was more active and stable than CalB covalently immobilized on activated silica supports, activated alumina supports, epoxy-activated sepharose, and tresylated sepharose. Native CalB loses activity exponentially in a first order deactivation pattern, while Novozym 435 shows a much slower deactivation pattern [77]. Due to its robustness and high activity, Novozym 435 is a promising biocatalyst for enzymatic glycolipid synthesis in DES (Table 2).

3.2 *Influence of Water Activity on Lipase-Catalyzed Transesterification*

Hydration of enzymes is important for their stability and activity [78–81]. However, for transesterification reaction almost anhydrous conditions are necessary in order to reverse the enzymes' activity from hydrolysis to esterification [82, 83]. Therefore, water activity is a crucial parameter in enzymatic glycolipid synthesis. Water removal systems were improving reaction yields of glucose fatty acid esters and trehalose diesters in different organic solvents with conversions up to 95% [12, 84, 85].

Novozym 435 is an enzyme widely applied in transesterification reaction due to its beneficial properties. Due to the immobilization of *Candida antarctica* lipase B on a hydrophobic polymeric resin, the carriers do not strip off water from the enzyme and a sufficient hydration level is possible also at low water content of the media [77]. In 2-methyl-2-butanol, highest glucose palmitate yields were reached at a water activity of 0.07, however at such low water content enzyme selectivity was reduced and the diester was produced as side product [31]. Lee et al. [33] reported an optimal water activity of 0.2 for transesterification reactions in ILs with Novozym 435, 0.4 with *Candida rugosa* lipase, and 0.5 with Lipozyme IM. At higher water activities the reaction rates decreased [33]. However, due to the strong hydrogen bond network, a defined water content is necessary for biocatalysis in DES in order to make substrates accessible. Low conversions of phenolic acids were observed without addition of water, while at 8–10% of water (water activity between 0.15 and 0.25) almost complete transesterification occurred [73]. Arabinose laurate yield in DES was significantly increased by an addition of 4% water compared to the reaction in DES without addition of water [86] and also sorbitol laurate conversion in DES was highest with addition of 5% water [37, 56].

3.3 *Influence of Sugar Loading on Enzymatic Glycolipid Synthesis*

Sugar solubility is rather poor in organic solvents applied for glycolipid synthesis, such as acetonitrile, acetone, t-butanol, hexane, or 2-methyl-2-butanol [85]. Ionic

Table 2 Conversions of different lipases in organic solvents, ionic liquids, and deep eutectic solvents

Solvent	Lipase	Reaction conditions	Conversion	Reference
Organic solvents	Novozym 435	Acetone, 45°C, 72 h, glucose palmitate, transesterification	93	[75]
		t-Butanol, 45°C, 72 h, glucose palmitate, transesterification	88%	[75]
		2-Methyl-2-butanol, 40°C, 72 h, fructose palmitate, esterification	53%	[35]
	Rhizomucor miehei	Acetone, 45°C, 72 h, glucose palmitate, transesterification	2%	[75]
		t-Butanol, 45°C, 72 h, glucose palmitate, transesterification	3%	[75]
		2-Methyl-2-butanol, 40°C, 72 h, fructose palmitate, esterification	30%	[35]
	Thermomyces lanuginose	Acetone, 45°C, 72 h, glucose palmitate, transesterification	28%	[75]
		t-Butanol, 45°C, 72 h, glucose palmitate, transesterification	32%	[75]
	Pseudomonas cepacia	Acetone, 45°C, 72 h, glucose palmitate, transesterification	–	[75]
		t-Butanol, 45°C, 72 h, glucose palmitate, transesterification	3%	[75]
Ionic liquids	Novozym 435	60°C, 72 h, glucose fatty acid esters, transesterification [BMIM][BF4]: t-butanol or [BMIM][PF6]: t-butanol (3:2)	59	[32]
	Rhizomucor miehei		8	
	Thermomyces lanuginose		33	
	Pseudomonas cepacia		<5	
	Candida rugosa		<5	
	Candida antarctica lipase A		<5	
Deep eutectic solvents	Novozym 435	50°C, 48 h, transesterification of *Millettia pinnata* seed oil, choline acetate: glycerol	55	[64]
	Lipozyme TLIM		45	
	Penicillium expansum		8	
	Novozym 435 Lipozyme TL IM CalA Immo 150 Lipase TL CLEA Lipozyme 435	50°C, 48 h, sorbitol laurate, transesterification, sorbitol: choline chloride	20 <10 <10 <10 20	[56]

liquids and DES are solvents with a wide range of different physical properties, so that in some, such as [Bmim][TfO] and hydrophilic DES, the sugar solubility is very good while in others it is as limited as in organic solvents [33, 36]. A limited sugar solubility and thus reactant availability can strongly influence the synthesis efficiency and is therefore a crucial parameter.

Flores et al. [85] showed that the dissolution of the excess sugar is not as fast as initial reaction rate in transesterification in 2-methyl-2-butanol [85]. Glucose dissolution rate was enhanced by crystalline ß-glucose and amorphous glucose resulting in higher dissolution rates and higher initial reaction rates. However, only for amorphous glucose a slightly higher yield was observed. A four times higher initial reaction rate and an 18% higher yield were achieved by the application of supersaturated glucose solution [85]. Acylation rates of disaccharides in organic solvents also depend on the dissolved sugar. Higher conversions were reported for disaccharides with a higher solubility. For the production of butanoate esters in tert-butanol yields were improved by using amorphous disaccharides compared to less soluble crystalline disaccharides [87].

Lee et al. [33] could correlate enzyme activity with the dissolved sugar concentration for glycolipid synthesis in ionic liquids [33]. Higher reaction rates and yields were achieved using supersaturated glucose solution than using saturated glucose solution in ionic liquids [33]. These results are in accordance with Shin et al. [88] who reported higher reaction rates, yields and productivities using supersaturated sugar solutions for glucose, fructose, and sucrose laurate synthesis in ionic liquids [88].

A beneficial effect of increased sugar amounts on initial reaction rates and yields was also shown in DES. Higher initial sugar addition resulted in a ninefold increase in glucose monodecanoate yield in a hydrophobic (−)-menthol: decanoic acid DES [28].

3.4 Influence of Fatty Acid Concentration on Transesterification Reactions

Inhibiting effects of high fatty acid concentrations were observed in transesterification reactions in organic solvents. Equimolar ratios of fatty acid and sugar led to highest yields in glucose myristate synthesis in organic solvents while fatty acid excess resulted in reduced conversions [89, 90]. An inhibitory effect of high fatty acid concentrations was also observed in other transesterification reactions catalyzed by *Candida antarctica* lipase B, *Candida rugosa* lipase, and *Rhizopus oryzae* lipase [91–96]. The inhibiting effect of fatty acids is due to the formation of non-productive complexes between fatty acids and the enzyme that are reported for reactions following ping-pong mechanism [91, 93, 96].

Lin et al. [97] reported an optimal fatty acid to sugar ratio of 1:5 for a biphasic system of ionic liquid and 2-methyl-2-butanol while productivity decreased with

higher fatty acid concentrations [97]. Ha et al. [98] investigated sugar to fatty acid ratio from 1:1 to 1:10 in ionic liquids with highest enzyme activity for an equimolar ratio of sugar and fatty acid [98]. However, Mai et al. [99] reported highest glucose laurate yields with an excess of fatty acid (sugar: fatty acid, 1:7.6) and also Galonde et al. [100] reported beneficial effects of a strong excess of fatty acid on mannosyl myristate synthesis in pure ionic liquids [100]. In ionic liquid with DMSO as cosolvent (DMSO:IL, 1:20) a sugar to fatty acid ratio of 3:1 resulted in highest conversions while at equimolar ratios or a greater excess of fatty acid yields decreased [101]. The difference in these studies might be explained by the fact that Ha et al. used free fatty acids and supersaturated sugar solutions in an esterification while Mai et al. and Galonde et al. used vinylated fatty acids and sugar concentrations below saturation in a transesterification reaction. Therefore, the mechanism of the reaction as well as the overall substrate loading differed between the studies limits their comparability. During esterification reaction water is released as a side product which shifts the reaction toward hydrolysis. While in transesterification ethenol is released which tautomerizes to acetaldehyde and evaporates. Thus, the reaction gets shifted toward transesterification and is, therefore, thermodynamically favored.

In DES, an inhibitory effect of excess fatty acid was observed similar to the studies in organic solvents [27].

While fatty acids show in general good solubility in the organic solvents applied in transesterification, fatty acid solubility is limited in many ionic liquids and deep eutectic solvents [27, 102]. Therefore, fatty acids are not necessarily dissolved in ionic liquids and DES, but fatty acid-solvent emulsions may be formed. This inherent difference between the solvent systems might also be an explanation for the varying observations in suitable fatty acid ratios for transesterification reaction.

3.5 Influence of Solvent Hydrophobicity and Nucleophilicity on Lipase-Catalyzed Transesterification

Furthermore, solvent hydrophobicity and nucleophilicity are parameters that are identified as crucial for transesterification reactions. For transesterification of 2-phenyl-1-propanol with vinyl acetate, transesterification rates were higher in more hydrophobic organic solvents: methyl-t-butyl-ether>hexane>toluene>tetrahydrofuran>acetonitrile>dimethylsulfoxide [80]. In organic solvents, higher sugar ester yields were achieved in less nucleophilic solvents. For transesterification using Novozym 435, Šabeder et al. [35] reported higher conversions in butanone and acetone than in t-butanol [35] and Bouzaouit and Bidjou-haiour [30] reported higher reaction rates in tetrahydrofuran and butanone than t-butanol [30]. t-butanol is more polar than butanone and tetrahydrofuran according to the solvatochromic parameter E_T^N [103]. The same pattern was observed using *Candida antarctica* lipase B, *Mucor miehei* lipase and *Pseudomonas*

cepacia lipase for lactose and sucrose ester synthesis, yields were higher in 2M2B than in acetone and lowest in methyl ethyl ketone [104]. Less hydrophilic solvents have lower ability to strip off water from the enzyme [79–81].

It has also been shown for ionic liquids that the enzyme activity depends on the properties of the solvent. For transesterification of benzyl alcohol with vinyl acetate, enzyme stability and enzyme activity was dependent on hydrophobicity of the ionic liquid used [105]. More nucleophilic ILs like [Bmin][TfO] enabled lower enzyme activity and stability than less nucleophilic, more hydrophobic IL [105]. In a transesterification study by Kaar et al. [106], enzyme activity in the ionic liquid [Bmim][PF6] was higher than in organic solvents [106]. However, no transesterification occurred by varying the anions resulting in more hydrophilic ILs. Re-suspension of the enzyme in water revealed that inhibition was reversible with acetate and methylsulfonate anions while nitrate anions exhibited irreversible inactivation of enzymes [106]. Immobilization could not enhance enzyme stability in hydrophilic ionic liquids [106]. Investigations of enzyme structure using IR analysis revealed a loss of the secondary structure of the enzyme in ionic liquids with ethyl sulfate, nitrate, or lactate anions [107]. In these solvents transesterification activity of Novozym 435 was strongly reduced, indicating that nucleophilicity, strong hydrogen bond accepting and donating properties of ionic liquids lead to reduced lipase activity [107]. Similar effects were also reported for transesterification of 2-phenyl-1-propanol with vinyl acetate: transesterification rates were higher in more hydrophobic ILs with higher reaction rates in [Emim][Tf2N] than in [C2OC1mim][Tf2N] and [C2OHmim][Tf2N] [80].

Ganske and Bornscheuer [32] reported no activity of *Candida antarctica* lipase B for synthesis of glycolipids in pure [Bmim][BF4]. However, a conversion of 59% to glucose laurate was achieved by adding t-butanol to the ionic liquid resulting in a two-phase system [32]. In the less nucleophilic ionic liquids [Bmim][TfO] and [Hmim][TfO], Zhao et al. [108] reported up to 26% conversion in pure ionic liquids [108]. In ionic liquids with the more nucleophilic anion methyl sulfate lower conversion was achieved even though sugars were highly soluble in that system [108]. Also for those ionic liquids, higher conversion rates were achieved after mixing with an organic solvent [108]. Lin et al. [97] reported also that ionic liquids with methyl sulfate anion showed low conversions, while conversions in ionic liquids were better with increasing hydrophobicity of the cations. In a comparative study with four different ionic liquids and their mixtures, highest productivities combined with a high lipase stability were reported for mixtures of hydrophilic and hydrophobic ionic liquids [33].

Effects of deep eutectic solvents are less thoroughly investigated than in organic solvents or ionic liquids. However, some similarities between DES, organic solvents, and ionic liquids could already be observed. Hollenbach et al. [27, 28] showed that an increased solvent hydrophobicity increases glycolipid yields and also initial reaction rates were higher in the hydrophobic (−)-menthol: decanoic acid DES than in hydrophilic ones [27, 28]. Full conversion to menthyl laurate was reported for transesterification reaction using *Candida rugosa* lipase in a hydrophobic menthol: lauric acid DES [109, 110].

Moreover, the anion of the hydrogen bond donor affected transesterification reactions in DES. Zhao et al. [108] investigated glucose laurate synthesis in two-phase systems of 2-methyl-2-butanol and DES. Almost no conversion was observed (Lipozyme TLIM and Novozym 435) in choline chloride: urea and choline chloride: glycerol-DES, neither with Novozym 435 nor with Lipozyme TLIM, while higher conversion rates were obtained in choline acetate based DES, which were nevertheless lower than 15% [108]. Also for biodiesel production, choline acetate based DES were better suited than choline chloride based ones [64]. Glycerol and ethylene glycol as hydrogen bond donor resulted in higher activity than urea or acetamide for the production of biodiesel [64]. It was suggested that the hydrogen bonding network of the polyols would have an activating effect on the enzyme by interacting with a serine residue [64]. Elgharbawy [111] demonstrated increased hydrolytic lipase activity in choline chloride based DES with sugars as hydrogen bond donor for porcine pancreas lipase, Novozym 435, Immobead 150, and *Rhizopus niveus* lipase, while *Candida rugosa* lipase and Amano lipase PS stayed unaffected [111]. Contrarily, malonic acid and glycerol as hydrogen bond donors showed some inhibitory effects [111]. Oh et al. [47] investigated lipase activity and lipase stability in various DES [47]. Lipase was more active in DES with an amide hydrogen bond donor than with a polyol hydrogen bond donor, but for lipase stability the relation was reversed [47]. Still, they could not identify a correlation between solvatochromic properties of the DES and lipase activity [47].

4 Conclusion

The selection of the reaction conditions is a crucial step in biotransformation. For lipophilization of polar substrates, some parameters could already be identified as decisive for synthesis success independent of the solvent type.

High sugar concentrations and the use of supersaturated sugar solutions were revealed as beneficial for transesterification yields in all solvent types. In organic solvents an equimolar ratio of sugar and fatty acids resulted in highest conversion rates as an excess of fatty acids might lead to inhibitory effects. For ionic liquids and deep eutectic solvents, there are still more studies necessary to provide clear evidence as the field of applicable ionic liquids and deep eutectic solvents is a widely diverse field and solubility of fatty acids in these solvents varies considerably.

Low water activity is necessary to prevent hydrolysis of the ester products in organic solvents, as well as in ionic liquids and deep eutectic solvents. However, a certain water addition is mandatory in deep eutectic solvents to allow for an efficient reaction.

Solvent nucleophilicity and solvent hydrophobicity were also crucial no matter what type of solvent was used. Selecting a solvent with low nucleophilicity promises the highest yields as no water will be stripped off from the enzyme and solvents of low nucleophilicity do not disturb enzyme structure. Nevertheless, comparative

studies with solvents of different nucleophilicity and hydrophobicity are still needed, especially for DES, as the currently available studies do not cover the broad spectrum of possible DES systems.

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Overview on Glycosylated Lipids Produced by Bacteria and Fungi: Rhamno-, Sophoro-, Mannosylerythritol and Cellobiose Lipids

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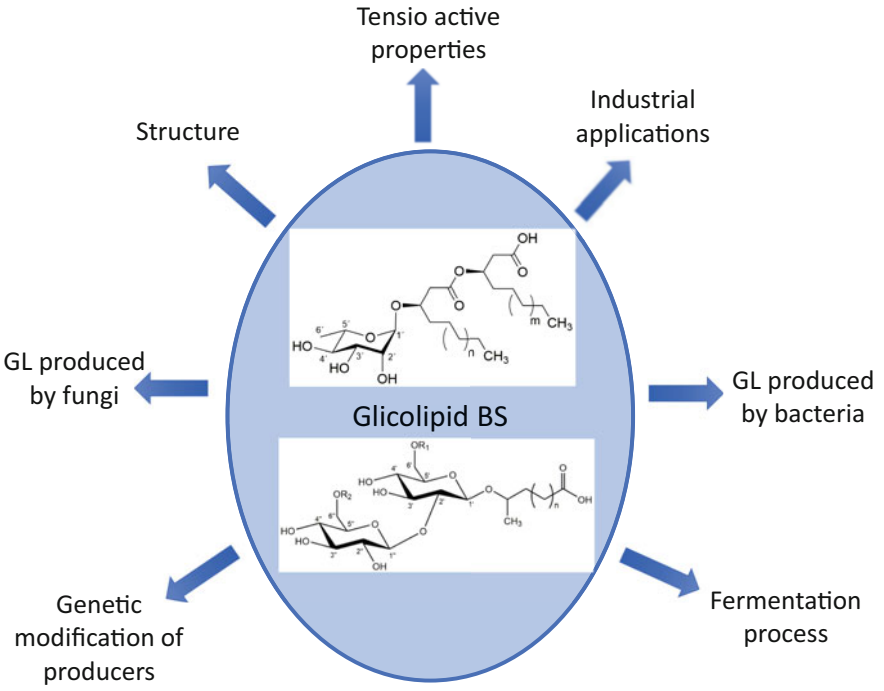
Abstract Wide ranges of microorganisms produce glycosylated lipids (GL), which are characterized by their tensio-active properties. Therefore, they can be used in different industrial applications as biosurfactants, such as food, agriculture, cosmetics, and health products among others. Two GL biosurfactants, rhamnolipids (RL) and sophorolipids (SL), are now commercially available and share a significant part of the biosurfactant market that in 2017 represented about 2.5% of the total surfactants market, estimated at 15 million tons globally.

In this chapter, we present a general overview of GL biosurfactants in terms of their diversity and the microorganisms that produce them. Additionally, we focus on the more detailed description of RL, SL, mannosylerythritol lipids (MEL), and cellobiose lipids (CL).

Pseudomonas aeruginosa, the ubiquitous opportunistic pathogenic bacterium, is the best RL producer, but other non-pathogenic bacteria like *Burkholderia thailandensis* and *Pseudomonas chlororaphis* NRRL B-30761 are also capable of producing them naturally. In addition, *Pseudomonas putida* has been used as heterologous host to produce RL with good yields. Here we describe the biosynthetic pathway for RL production, the genes involved in its synthesis, and some of the challenges for producing a homogeneous RL product in high quantities that is suitable for specific applications.

SL, MEL, and CL are some of the GL biosurfactants produced in high quantities by fungi, like *Starmerella bombicola, Moesziomyces aphidis,* or *Ustilago maydis*. We provide an overview of some of their characteristics, insights on the metabolic pathways involved in their synthesis and genetic modifications performed to increase their production, as well as fermentation and purification strategies and some of their applications.

Graphical Abstract



Keywords Cellobiose lipids, Mannosylerythritol lipids, *Pseudomonas*, Rhamnolipids, Sophorolipids, *Starmerella bombicola*, Ustilaginaceae

1 Glycolipid Biosurfactants Produced by Bacteria

1.1 General Characteristics of GL Produced by Bacteria

Simple GL produced by bacteria have tensio-active properties since they are composed of a sugar moiety that is hydrophilic and a lipid part that is hydrophobic. It has been reported that different bacteria produce hundreds of GL. The bacterial genera that produce the higher number of these compounds are Actinobacteria, followed by Proteobacteria [1].

GL produced by bacteria are believed to play a biological role due to their antimicrobial activity determined by its surface-active properties, such as promoting the competition with other microorganisms [1]. Thus, these compounds have potential in industrial applications in several areas. However, in most cases they are produced at such a low amount that it is not feasible to produce them at an industrial scale.

1.2 RL General Characteristics and Industrial Applications

Despite the high diversity of GL produced by bacteria, the most studied of these glycolipid biosurfactants is rhamnolipids (RL). This is due to its excellent properties (Table 1) for different industrial applications [28–30], its low toxicity [31], and high biodegradability. Additionally, because RL is produced at a higher level compared with other bacterial GL, it can be produced at around 100 g/L in industrial conditions. At present, RL that are in the market are primarily used in the petrochemical industry, bioremediation of different pollutants, household products, agricultural chemicals, and personal care products [30]. In addition, RL present other activities such as antifungal properties [32, 33], antimicrobial activity, and they show low toxicity and do not disturb the immune response, so these characteristics expand its applications in pharmaceutical and therapeutic industries. RL potential industrial uses go from cosmetic, antimicrobial, and antibiofilm agents and other uses in biomedicine [34–37], agricultural and composting [38–40], and environmental remediation [41, 42], among others. These GL biosurfactants have reached the market in the last decade and nearly 95,000 tons were produced in 2013 representing nearly 455 millions of US dollars but there are some challenges to be solved in order for RL representing a big share of the surfactant market. These challenges will be highlighted in this chapter.

Table 1 Surface tension and SMS of several RL, SL, MEL, and CL variants

Strain	Main influencing C-source	GL	Surface tension [mN/m]	CMC	References
P. aeruginosa KT1115[a]	Rapeseed oil[a]	Mono-RL	~28	167 mg/L	[2]
P. aeruginosa KT1115[a]	Rapeseed oil[a]	Di-RL	~28	8 mg/L	
C. floricola TM 1502	Olive oil, oleic acid, postmodificated	Acidic SL-diacetylated	39.8	170×10^{-6} mol/L	[3]
		Acidic glucose lipid GL, acetylated	47.3	170×10^{-6} mol/L	
S. bombicola ATCC 22214, WT and different modified strains	Rapeseed oil	Lactonic SLs	33.9	45.1 mg/L	[4]
		Non − +diacetylated acidic SLs	38.2	112 mg/L	
		Non-acetylated acidic SLs	40.9	245 mg/L	
S. bombicola ATCC 22214	Palmitic acid	92% lactonic SL	35	200 mg/L	[5]
	Stearic acid	99% lactonic SL	35	35 mg/L	
	Oleic acid	100% lactonic SL	36	140 mg/L	
	Linoleic acid	98% lactonic SL	36	250 mg/L	
	2-dodecanol, SL was postmodificated with enzymes	SL-alcohol ($SL\text{-}E_{2-12}$)	31	200 mg/L	[6]
		GL-alcohol, one sugar cleaved ($GL\text{-}A_{2-12}$)	31	200 mg/L	
		SL-alcohol with 2 [a]hydroxy-C10 at C6' and C6''; ($SL\text{-}E_{2-12}$di-3-OH-C10)	27	200 mg/L	
M. antarcticus T-34	Soybean oil	MEL-A	28.4	2.7×10^{-6} mol/L	[7–9]
		MEL-B	28	4.5×10^{-6} mol/L	
	Glucose	Mono-acylated MEL	33.8	3.6×10^{-4} mol/L	[10]
M. antarcticus KCTC7804	Soybean oil	MEL	29	15.8×10^{-6} mol/L	[11]
M. antarcticus JCM 10317	Sucrose	MEL-A	25.3	3.6×10^{-6} mol/L	[12]

M. aphidis DSM 70725	Soybean oil	MEL	26.2	–	[13, 14]
T. crassus CBS9959	Oleic acid	MEL-A	26.5	5.2×10^{-6} mol/L	[15]
U. maydis NBRC 5436	Sucrose	MEL-A	28.6	2.9×10^{-6} mol/L	[12]
M. Parantarcticus JCM11752	Glucose/olive oil and mannitol	MML	24.2	2.6×10^{-6} mol/L	[16]
	Olive oil and ribitol	MRL	23.7	1.6×10^{-6} mol/L	[17]
	Olive oil and arabitol	MAL	24.2	1.5×10^{-6} mol/L	[17]
P. tsukubaensis NBRC1940	Castor oil	MEL-B additional OH	28.5	2.2×10^{-5} mol/L	[18]
P. tsukubaensis JCM16987	Olive oil, arabitol	MAL-B	26.1	1.2×10^{-5} mol/L	[19]
U. scitamineum NBRC 32730	Sucrose	MEL-B	25.2	3.7×10^{-6} mol/L	[12, 20]
S. graminicola CBS 10092	Soybean oil	MEL-C	24.2	4×10^{-6} mol/L	[21]
P. hubeiensis KM59	Soybean oil	MEL-C	25.1	6×10^{-6} mol/L	[22]
P. hubeiensis Y10BS025	Soybean oil	MEL-C	30.8	–	[23]
P. hubeiensis SY62 after deletion of *emt1*	Olive oil	MEL-D	29.6	2×10^{-5} mol/L	[24].
U. siamensis CBS 9960	Sucrose	MEL-C	29.8	6.4×10^{-6} mol/L	[12]
	Safflower oil	MEL-C	30.7	4.5×10^{-6} mol/L	[25]
C. humicola 9–6	Glucose	CLs	37 in 0.1 $NaHCO_3$	2×10^{-5} mol/L	[26]
C. humicola JCM1461	Glucose	CLs	–	pH 4: 3.3×10^{-5} mol/L pH 7: 4.1×10^{-4} mol/L	[27]

[a]The main congener of mono-RL and di-RL produced by all P. aeruginosa strains contains C10-C10 HAAS; the nature of RL produced is not greatly influenced by carbon source used in culture medium. We report strains and medium used by Zhou et al. [2]

Several reviews about different aspects of RL research have been published (see, for example, [41, 43–46]). Hence, we will only focus on the discussion of some aspects of RL production that are currently being studied.

P. aeruginosa, the bacterium that produces the highest RL yields, mainly produces two types of RL, the one that contains one rhamnose moiety called mono-RL, and the one containing two rhamnose molecules called di-RL (Fig. 1, Table 1). In turn, these two types of RL have different congeners with respect to their lipidic part, being the α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rha-C10-C10) and α-L-rhamnopyranosyl-(1–2)-α-L-rhamnopyranosyl-3-hydroxydecanoyl-3-hydroxy-decanoate (Rha2-C10-C10), the most abundant congeners. However, smaller proportions of molecules containing C12-C10, C12-C12, and C10-C12:1 dimers are also produced [47]. Most *P. aeruginosa* isolates produce a mixture of mono- and di-RL, but those strains that belong to the PA7 clade, such as strain ATCC 9027, produce only mono-RL [48].

Physicochemical characteristics of mono- and di-RL are different (Table 1) [2], thus the characteristic of the biosurfactant produced by different *P. aeruginosa* strains will depend on the molar ratio of these two types of RL. The di-/mono-RL molar ratio is characteristic of each *P. aeruginosa* isolate and shows little variation in different culture conditions [2].

1.3 RL Synthesis and Regulation in P. aeruginosa

The best RL producer is *P. aeruginosa*, a γ-proteobacterium that is on the one hand, a widespread environmental bacterium [49, 50], and on the other hand, also an important opportunistic pathogen [51]. The pathogenicity of *P. aeruginosa* represents a serious limitation for its industrial use for RL production, especially because the production of this biosurfactant is regulated at the transcription level by the so-called quorum-sensing (QS) response that regulates the expression of most of its virulence-associated traits [52]. This co-regulation may be due to the participation of RL in processes that are important for the establishment of *P. aeruginosa* infections [53], such as swarming motility [54].

P. aeruginosa QS is a complex regulatory hierarchical cascade that includes three transcriptional regulators LasR, RhlR, and PqsR that activate transcription when coupled with their cognate autoinducer 3-oxo-dodecyl homoserine lactone (C12-HSL), butanoyl homoserine lactone (C4-HSL), or 2-heptyl-3,4-dihydroxyquinoline (PQS), respectively [55, 56]. Genes involved in the production of mono-RL (*rhlAB*) [57] or di-RL (*rhlC*) [58], as well as those involved in the synthesis of dTDP-L-rhamnose (*rmlBDAC*) [59], the precursor of mono- and di-RL, are regulated by RhlR/C4-HSL. The expression of *rhlR* is under a positive autoregulatory loop, since it can form an operon with the upstream-encoded *rhlAB* operon under conditions of high RL production [60], such as temperatures above 37°C [61]. Thus, this *P. aeruginosa* genetic circuit permits the coordinate induction of genes involved in RL production.

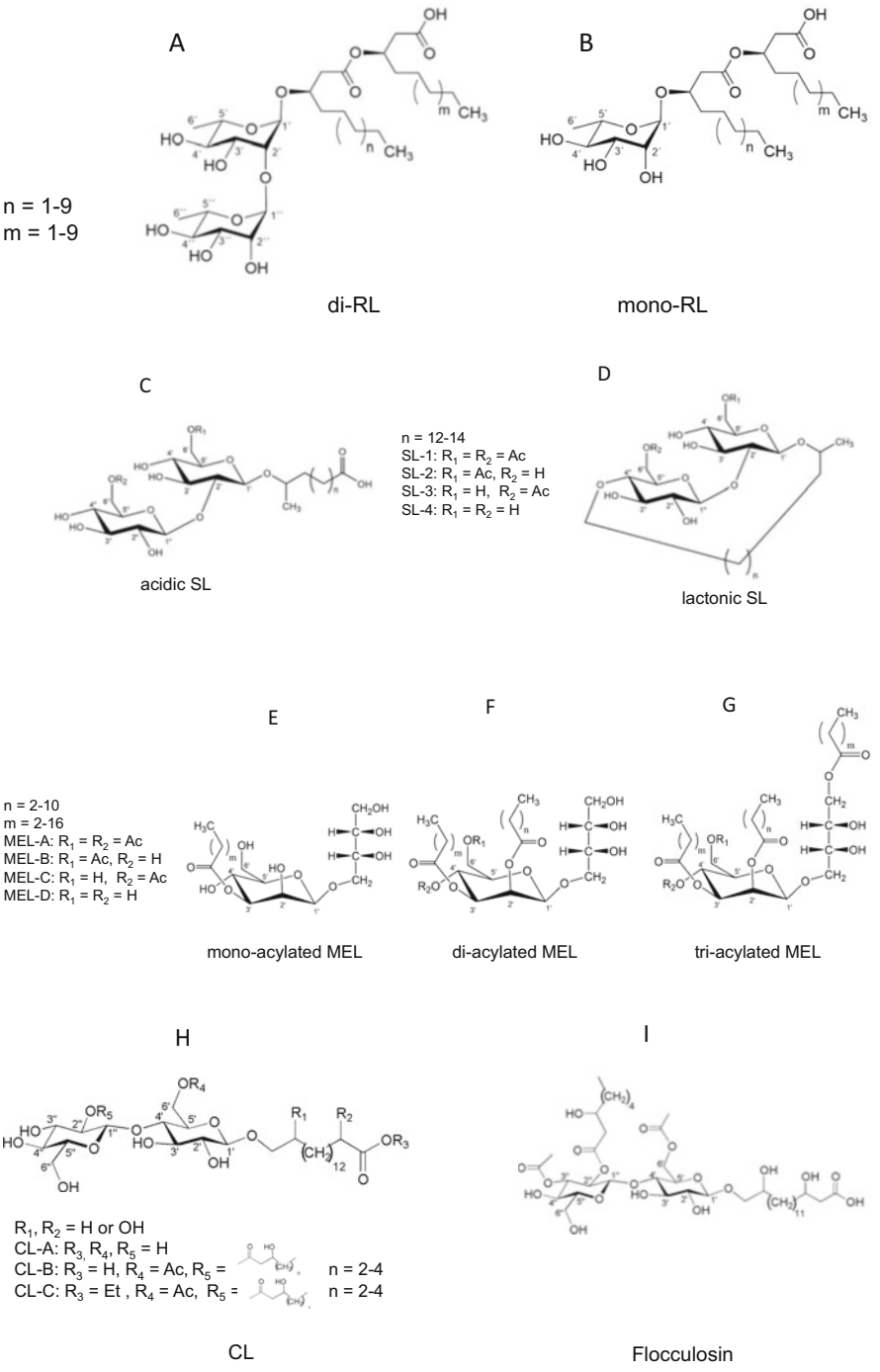


Fig. 1 Chemical structures of the glycolipid biosurfactants described in this work. (**a**) monorhamnolipid produced by *P. aeruginosa*, (**b**) di-Rhamnolipid produced by *P. aeruginosa*, (**c**) acidic sophorolipid (**d**) lactonic sophorolipid (**e**) mono-acylated mannosylerythritol lipid (**f**) diacylated mannosylerythritol lipid (**g**) triacylated mannosylerythritol lipid (**h**) cellobiose lipid (**i**) flocculosin

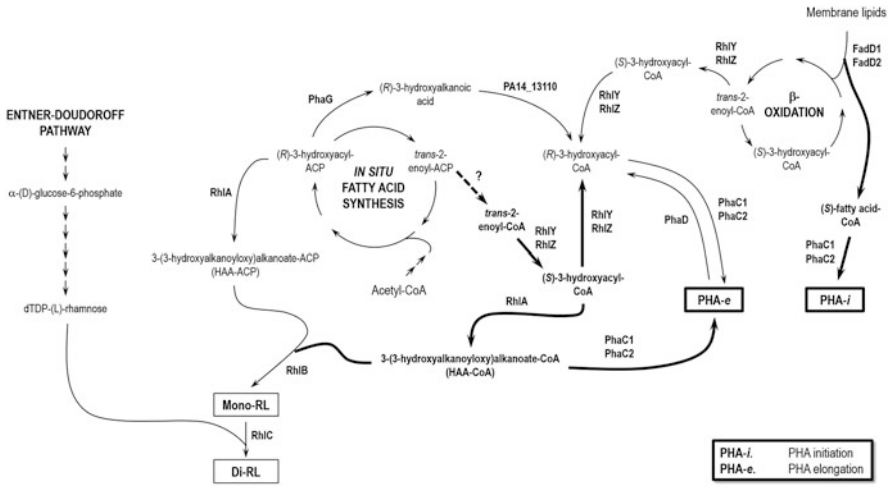


Fig. 2 Schematic representation of *P. aeruginosa* PA14 RL biosynthetic pathway (modified from [62])

1.4 P. aeruginosa *RL Biosynthesis Is Interrelated with the Synthesis of Polyhydroxyalkanoates (PHA)*

P. aeruginosa RL biosynthetic pathway is very intricate and the precursors of this biosurfactant are derived from central metabolic pathways (Fig. 2). Mono-RL is produced by the coordinated activity of RhlA that uses mainly Co-A linked fatty acids produced by RhlY and RhlZ [62, 63] to synthetize 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), mainly 3-hydroxydecanoyl-3-hydroxydecanoate, that are used as precursor by RhlB to produce mono-RL. In turn, RhlC uses mono-RL to produce di-RL [58]. Rhamnosyl transferases RhlB and RhlC use as rhamnosyl donor dTDP-L-rhamnose that is synthetized from glucose 6-phosphate by AlgC [64] RmlA, RmlB, RmlC, and RmlD enzymes [59].

P. aeruginosa produces polyhydroxyalkanoates (PHA) as carbon storage, which are constituted mainly of C10 fatty acid monomers. PHA synthesis diminishes the production of RL, since the synthesis of this fatty acid polymer competes at several points for fatty acids precursors (Fig. 2). Furthermore, it has been reported that RhlA produces PHA intermediates [62], so when either of the PHA synthases PhaC1 or PhaC2 are active, a portion of the fatty acid precursors of RL will be drained to PHA synthesis even in the absence of the PhaG thioesterase [65].

1.5 Other Bacteria That Produce RL

Pseudomonas aeruginosa is not the only bacterial species that produces RL, but it is the best producer [66]. Several *Burkholderia* species, including some that are

non-pathogenic such as *B. thailandensis* [67] and *B. kururiensis* [68], can produce mainly di-RL, but with a lipid moiety of a HAA of C14-C14 fatty acids.

In addition, strain NRRL B-30761 of the non-pathogenic *P. chlororaphis* is able to produce mono-RL [69, 70] and has been engineered to produce also di-RL by the expression of *P. aeruginosa rhlC* [71, 72].

A non-pathogenic marine bacterium (*Marinobacter sp* MCTG107b) was reported to produce a mixture of RL, with over 95% of di-RL, being di-RL with a lipidic dimer of C10-C10, the most abundant congener [73].

These non-pathogenic RL-producing bacteria are an important resource for the industrial production of RL, but a large amount of work remains to be done with them to attain a much higher RL productivity.

The screening of different environments for non-pathogenic bacteria that produce high levels of biosurfactants, specifically of RL, that might be used in different industrial applications is an important research area. The production of biosurfactants by marine bacteria was reviewed, but none avirulent RL-producing bacterium was mentioned [74].

1.6 Bioengineering Strategies for RL Production

Several aspects of RL production have been studied to achieve the large-scale production of this GL biosurfactant with an appropriate cost for its industrial application [45, 75, 76]. One of the main problems to produce RL and other biosurfactants in bioreactors is foaming with the subsequent loss of biomass. To cope with this situation, different strategies have been followed [44, 77, 78]; within these strategies, RL have been produced under microaerophilic conditions using nitrate as an electron acceptor, with good results [79].

It has been recognized that RL production is enhanced when *P. aeruginosa* is cultivated on media that have a limitation of phosphorous [80, 81], or with nitrate as nitrogen source [82], and using glucose, glycerol, or vegetable oils as carbon sources [83, 84]. The effect of the use of glucose as carbon source plus fatty acids of different length for high levels of mono-RL production by strain ATCC 9027 was reported [85]. Culture media have been devised using optimization models to optimize RL production [86–88]. In addition, several renewable or waste products have been evaluated as substrates to produce RL at lower cost [28, 89–92].

1.7 Downstream Processing of RL

The purification of biosurfactants is costly, as this process can represent more than a half of the production cost. This is especially the case when they are applied in the cosmetic or biomedical industries where a high purity level is required.

In the case of RL, the advantage is that it can be produced using glucose [93] or glycerol [94] as carbon sources. As a result, this allows the purification step from culture supernatants to occur without solvent extraction to separate this biosurfactant from oils used as fermentation substrates. The most common procedures for RL extraction are foam fractionation and adsorption [95–97]. An efficient method using silica gel column chromatography for the separation of mono- RL from di-RL was reported [2].

1.8 Genetic Engineering Strategies to Build Bacterial Strains with Enhanced RL Production

In general, two strategies can be distinguished to construct bacterial strains that produce higher RL levels. One of these strategies uses natural RL producers, mainly *P. aeruginosa*, to build genetic engineered strains that have an enhanced production of this biosurfactant. Yet, the other strategy uses non-pathogenic bacteria as heterologous host for the expression of genes involved in RL production.

In the case of the genetic manipulation of *P. aeruginosa* strains, it has been reported that the expression from a plasmid of the *rhlAB-R* operon in the avirulent ATCC 9027 strain causes an increased mono-RL production reaching a level comparable with RL (a mixture of mono- and di-RL) produced by the PAO1 type strain [98]. It has also been reported that a PA14 derivative with a redirected carbon-flow to RL production by the complete blockage of PHA synthesis and expressing from a plasmid the *rhlAB-R* operon produces considerably more RL than the original PA14 strain, and nearly two times the RL produced by PAO1 strain [65]. The main advantage of the genetically manipulating of *P. aeruginosa* strains to enhance their RL for developing strains suitable for large-scale production is that genes involved in the synthesis of this biosurfactant are coordinately induced by RhlR/C4-HSL which is positively auto-regulated. Another advantage is that this bacterium expresses the enzymes RhlY and RhlZ that produce the fatty acid intermediates of RL synthesis that have been used as heterologous host for RL production in other bacterial models where they are absent. In addition to the genetic manipulation of the QS-regulated genes, it was reported that the overexpression of *estA* encoding for an esterase in *P. aeruginosa* cultivated in a simple medium increased RL production [99].

Several bacterial models have been used as heterologous hosts for RL production, and the best results were obtained using *P. putida* to produce mono-RL by expressing *P. aeruginosa rhlAB* operon [94, 100, 101]. In the case of *P. putida* KT2440, a significant RL production was achieved using a derivative that expressed the *rhlAB* operon from an inducible promoter and contained a partial blockage of PHA synthesis [101]. The expression of the *rhlAB* operon together with *rhlC* in *P. putida* KT2440 leads to the production of both mono- and di-RL, but the level of their production is low [102]. The optimization of medium composition and

fermentation conditions for heterologous mono-RL production by *P. putida* KT2440 has been reported [77, 78] and the production of significant titers of this biosurfactant has been achieved (Table 2) [95]. The *rhlAB* operon of *Burkholderia glumae* was expressed in *P. putida* KT2440 to produce RL with long-length fatty acid moieties, mainly C14-C14 [119].

The production of RL using *Escherichia coli* has been reported, but only a very low production was achieved and only when the *rmlBDAC* operon was expressed together with the *rhlAB* operon, showing the importance of dTDP-L-rhamnose availability for the synthesis of this biosurfactant [120]. The expression in *E. coli* of *P. aeruginosa rhlAB* operon and *rhlC*, and the site directed mutagenesis of *rhlB* permitted the production of modified RL that were said to be useful for enhanced oil recovery [121]. In addition, the expression in *E. coli* of the codon-optimized *P. aeruginosa* and *B. pseudomallei rhlAB* operon and *rhlC* in different combinations and under the control of different promoters was investigated and the production of mainly di-RL congeners was attained [122].

The best-characterized GL biosurfactants are still RL, but as will be described, they are produced at a considerably lower concentration than GL biosurfactants produced by fungi, even when they are produced by *P. aeruginosa,* the best RL producer (Table 2).

2 Glycolipid Biosurfactants Produced by Fungi

2.1 Different Strains to Produce Fungal Glycolipids

Besides bacteria, there are many fungi known for their production of simple glycolipids, especially fungi from the phylum Ascomycota and Basidiomycota. Ascomycota include species like *Komagataella phaffii* (initially referred to as a *Pichia pastoris), Sordaria macrospora, Rhynchosporium secalis,* and all of them have been described to produce glycosylated sterols [123]. Polyketide glycosyde are produced by *Gliocladium catenulatum* [124–128] or Clonostachys candelabrum [129], whereas glycosyl- and mannosyl-lipids are produced by *Aspergillus niger* [130], *Fusarium sp.*[131], and *Simplicillium lamellicola* [132] and Liamocins, polyol fatty acid esters by *Aureobasidium pullulans*. MEL lipids are synthesized by *Geotrichum candidum* [133].

The most prominent strain to produce SL is *Starmerella bombicola* (initially referred to as *Torulopsis or Candida bombicola*), discovered by Gorin et al. [134] [135]). SL synthesis is also described for *C. batistae* [136, 137], *C. floricola* [3, 138], *C. apicola* [138], *C. riodocensis* [139], *and C. stellata* [140]. Furthermore, SL synthesis is described for *C. kuoi* [140, 141], *Candida albicans O-13-1* [142], *C. rugosa* [143], *C.* tropicalis [143], *Cyberlindnera samutprakarnensis* [144], and *Lachancea thermotolerans* [145]. For *Wickerhamiella domercqiae* [146, 147] SL synthesis was shown, however the strain was later reclassified to *S. bombicola*

Table 2 Overview of fermentation processes for the production of RL, SL, MEL, and CL[a]

Strain	$V_{fermenter}$ process	C-source	Medium	Product	c [g/L] (time)	$Y_{X/S}$ [g/g]	r [g/(L h)]	References
Rhamnolipids								
Pseudomonas aeruginosa PAO1	30 L, batch, 37°C	Sunflower oil	Mineral salts with nitrate	Mono- (Rha-C10-C10) and di-RL (Rha_2-C10-C10)	36.7 ± 1.2 (5 days)	2.22 ± 0.42	0.43	[84]
Pseudomonas aeruginosa DSM 7108	30 L, batch, 30°C	Sunflower oil	Mineral salts with nitrate	Mono- (Rha-C10-C10) and di-RL (Rha_2-C10-C10)	35.7 ± 2 (5 days)	2.23 ± 0.28	0.26	[84]
Pseudomonas sp DSM 2874	30 L, batch, 37°C	Sunflower oil	Mineral salts with nitrate	Mono- (Rha-C10-C10) and di-RL (Rha_2-C10-C10)	30.8 ± 1.5 (5 days)	0.86 ± 0.14	0.35	[84]
Pseudomonas putida KT2440/ pSynPro8oT_*rhlAB*	Fed batch, 30°C	Glucose	Mineral salts	Mono-RL (Rha-C10-C10)	14.9 (80 h)	10 mg/G	18 mg/ g h	[78]
Sophorolipids								
Starmerella bombicola ATCC22214	2.5-L, fed batch	Glucose, soybean oil	Minerals, yeast extract	SL	80 (~4 days)	0.37 (batch) /0.6 (fed batch)	~1.7	[103]
	50-L, fed batch	Glucose, oleic acid or soybean or rapeseed oil	Yeast extract, urea or ammonia, corn steep liquor	SL with C18:0, C18:1, C18:2 in the fatty acid chain with oleic acid, C18:0, C18:1, C18:2; 18:3 with soybean oil, lactonic, deacetylated acid form	180 (~5 days)/ >300 g/L	~0.87 / 0.68	~1.4 / 2.4	[104, 105]
	4-L, fed batch	Rapeseed esters, glucose	Minerals, ammonia, corn steep liquor	SL	317 (~6 days)	0.65	~2.1	[106]
	3-L, fed batch	Single cell, rapeseed oil,	Yeast extract, ammonia	SL	422 (~17 days)	0.84	0.8	[107]

		glucose, lactose						
	10-L, fed batch, high cell density (80 g $_{DW,cells}$/L	Glucose, rapeseed oil	Yeast extract, malt extract, peptone, dextrose	SL	–	–	8.3 (with 15 units of media)	[108]
	3-L, fed batch	Glucose, oleic acid	Yeast extract, minerals	Non-, mono-, diacetylated SL; mainly C18:1; 57% lactonic SLs	114 (reached in ~2 days)	0.46	2.4	[4]
Starmerella bombicola NRRL Y17069	1.5-L, fed batch, resting cells	Glucose, sunflower oil	Yeast extract	SL	120 (8 days), resting cell	0.6	0.6	[109]
Pseudohyphozyma bogoriensis ATCC18809	14-L, fed batch	Glucose	Yeast extract, malt extract, peptone, dextrose	SL with C22 fatty acid; glucose depletion correlates with deacetylation	51 (13 days)	–	0.16	[71, 72]
Mannosylerythritol lipids								
Candida sp. SY16 (*P. tsukubaensis*)	5-L, batch	Soybean oil	Minerals, peptone	Diacylated MEL-B, fatty acid residues C6. C12. C14: 0, C14:1	–	–	–	[110]
M. aphidis DSM14930	72-L, fed batch	Soybean oil, glucose	Minerals, yeast extract	Diacylated MEL-A,B,C,D	165 (~12 days)	0.92	0.6	[111]
M. aphidis DSM 70725	72-L, fed batch	Soybean oil	Minerals, yeast extract	Diacylated MEL-A,B,C,D	70 (~9 days)	0.67	0.3	[111]
	15-L, fed batch	Rapeseed oil	Minerals, yeast extract	Diacylated MEL-A,B,C,D, triacylated MEL, fatty acid residues C8-C12 at C2' and C3'	69 (~18 days)	0.51	0.16	[112]

(continued)

Table 2 (continued)

Strain	$V_{fermenter}$ process	C-source	Medium	Product	c [g/L] (time)	$Y_{X/S}$ [g/g]	r [g/(L h)]	References
Candida sp. SY16 (*P. tsukubaensis*)	5-L, fed batch	Soybean oil, glucose	Minerals, peptone	Diacylated MEL-B	95 (~8 days)	0.45	0.02	[113]
S. Scitamineum NBRC32730	1.5-L, batch	Sugarcane	Yeast extract, peptone, urea or nitrate	Monoaceylated and diacylated MEL-B, fatty acid residues C8,C10, C12, C14:1; erythritol	25,1 (~7 days)	0.13	0.15	[20]
Cellobiose lipids and mannosylerythritol lipids								
U. maydis PRL-119 (initially *U. zeae)*	5-L, batch	Glucose	–	CL	22 (~2 days)	0.33	0.46	[114]
U. maydis ATCC 14826	20-L, fed batch, resting cells	Coconut oil,	N-limitation	Fatty acid residue C6,C12, C14, two further hydroxyl groups at C16 fatty acid	7.65 crude extract GL (~2 days)	0.59 (N-limit.), 0.79 resting cells	0.16	[115]
	Fed batch, resting cells	Glucose	N-limitation	Crude extract	–	0.25 (N-limit.), 0.66 resting cells	–	[115]
U. maydis L8	2-L, batch, fed batch	Glycerol	Minerals, ammonium, amino acids, vitamins	MEL and CL Fatty acid residues: C16 was 2.5 higher with glycerol compared to glucose as feedstock	32.1 GL (~8 days)	–	–	[116]
C. humicola JCM1461	5-L, repeated batch	Glucose	Peptone, yeast, malt extracts	CL, acetylation at 2″, 4″, 6′, one further hydroxy-group in C16 fatty acid	13 (~11 days)	~0.066	~0.05	[27]

U. maydis DSM 17144, 17,145 (mutant), DSM17146 (mutant)	1-L, resting cells	Sucrose. Glucose	Minerals, pH 2.5	Dependent on the strain CL/MEL; MEL or CL	7–33	–	Up to 0.01	[117, 118]

[a]Most abundant variants are underlined

[148]. A deeper overview for SLs is given in the review by Claus and Van Bogaert [149] and Roelants et al. [150].

Within the phylum of Basidiomycota *Rhodotorula glutinis* and *R. graminis* are described to produce mannitol and pentitol esters of 3-D-hydroxypalmitic and 3-D,-hydroxystearic acids. Synthesis of SLs is described for *Rhodotorula mucilaginosa* [143], *R. babjevae* [151], *Pseudohyphozyma bogoriensis* (initially referred to as *Candida,* then *Rhodotorula)* [152, 153], and *Cryptococcus sp.*[154] like *Cryptococcus curvatus* [155].

CLs were first found in *Ustilago zeae* [114, 156], which was renamed to *Ustilago maydis* and characterized from Frautz et al. and Spoeckner et al. [115, 157]. CL is also synthesized by *Cryptococcus humicola* [26], *Sympodiomycopsis paphiopedili* [158], *Anthracocystis flocculosa* [159], *Sporisorium graminicola* [160], *Kalmanozyma fusiformata* [161], *Trichosporon porosum* [162], and *Sporisorium scitamineum* [163–165].

MELs are known to be produced by *U. maydis* [157, 166], *Kurtzmanomyces* [167], *Dirkmeia churashimaensis* [168], *Ustilago cynodontis* [169], *U. shanxiensis* [170], *U. siamensis* as well as by [25], *Sporisorium scitamineum* [164], *Moesziomyces antarcticus* [171], *M. parantarcticus* [172], *M. aphidis* [111], *M. rugulosus* [173], *Kalmanozyma fusiformata* [174], *Pseudozyma tsukubaensis pro tem.*[1] [175], *P. hubeiensis pro tem.* [22], *Sporisorium graminicola* [160], and *Triodiomyces crassus* [15]. For some of the basidiomycetes mentioned above both metabolic pathways to produce MEL and CL are present. We would like to point out that many of the strains have been reassigned [176]. For consistency reasons, we used this latest nomenclature for the biosurfactants producer in this review. A comparison of the names before and after the renaming of MEL producers is very well presented by Beck et al. [177].

2.2 Structural Variety of SL, MEL, and CL in Wild-Type Strains

In the basic structure of SLs (Fig. 1), the hydrophilic group consists of sophorose, a disaccharide of two β-1,2-glycosidically linked glucose molecules that can be acetylated on the C6'- or/and C6"-position. As hydrophobic tail, a terminal (ω) or subterminal (ω-1) hydroxylated fatty acid with usually16–18 carbon atoms is β-glycosidically bound (i.e., via an ether bond) to the sophorose molecule [178, 179]. There are also examples for fatty acid chains with 22 carbon atoms shown in wild-type strains, like SLs produced by *Pseudohyphozyma bogoriensis* [71]. The hydroxylated fatty acid can be saturated or unsaturated, having one or more double bonds, depending on the oil or fatty acid used as feeding source. SL can

[1] *pro tem.* = pro tempore: The strains have been reclassified, but since they have not yet received a new clade name, they are referred to by the old name with the addition *pro tem.*

either take an open, so-called acid form in which the carboxyl group of the fatty acid is present as free fatty acid, or the fatty acid can be intramolecularly esterified so that the carboxyl group at the C4" position forms a lactone structure (Fig. 1). SL can also form lactones between two SL molecules [152]. Strains usually produce a mixture of several molecule variants, but specific variants are often associated to each microorganism. An overview of commonly produced structures is shown in Table 3.

The basic structure of MEL consists of a hydrophilic sugar head group 4-O-β-D-mannopyranosyl-D-erythritol to which usually two fatty acids with a chain length between 2 and 18 carbon atoms are linked via ester bonds to the mannose C2' and C3' position. Depending on the carbon source there are also some strains producing rare MELs, where small amounts of monoacylated or triacylated MELs are found. An overview of the molecule variants is also given in Table 3. Besides the variety in fatty acid residues, the mannose molecule within MEL can be acetylated at C4' and/or C6'position (Fig. 1) [196]. Generally, MELs are synthesized as a complex mixture of different chemical variants. The classification of the main variants MEL-A, MEL-B, MEL-C, and MEL-D refers to the degree of acetylation, which leads to different polarity and thus to different elution behavior on silicate-coated thin-layer chromatography (TLC) [171]. MEL-A is the diacetylated form, while MEL-B and MEL-C are monoacetylated at positions C6'or C4' respectively, and MEL-D is completely deacetylated. The group of Kitamoto and Morita reported in a series of articles the favored production of different strains, where *Moesziomyces rugulosus*, *M. aphidis,* and *M. parantarcticus* mainly produce MEL-A [16, 173, 174], *Sporisorium scitaminea* secrete MEL-B [12], *M. antarcticus* synthetize MEL-A and MEL-B and low amounts of MEL-C [171], *P. tsukubaensis pro tem.* preferably produce the diastereomer of MEL-B [175], and *S. graminicola, P. hubeiensis pro tem., Ustilago shanxiensis,* and *U. siamensis* mainly MEL-C [21, 22, 25, 170]. The molecule variation is also influenced by the number, length, and saturation of the fatty acid side chains, by the sugar configuration of the erythritol (S to R form) and mannosyl or by its exchange with other alditols (e.g., mannitol). The modification of individual MEL structures or the MEL mixture is mainly determined by the strain and its metabolism. However, also the saturation of the fed oil may have an impact on the fatty acid introduced into the MEL molecule. Beck et al. showed that different oils (soybean, canola, olive, coconut oil) do not significantly affect the acetylation pattern or fatty acid chain length, whereas the amount of double bonds in the substrate oil can be reflected in the MEL product. This was revealed in species that introduce a long-chain C14-C16 fatty acid chain into MEL, like *U. siamensis* or *U. shanxiensis* [187]. CL production was displayed for several MEL producing strains. For *M. aphidis* and *P. hubeiensis pro tem.* a low amount of CL was detected at nitrogen limiting conditions [190]. It is assumed that both pathways for MEL and CL are present in most of the MEL producing strains. Nevertheless, this is not yet proven for all strains.

The hydrophilic group in CLs is represented by cellobiose, a disaccharide with two glucose molecules linked by a β-1,4-glycosidic bond. Cellobiose is linked via an O-glycosidic bond (or ether bond) on C1'- position to the fatty acid residue in ω-position. This O-substituent could differ in the number of hydroxy-groups in the

Table 3 SL, MEL, and CL variants produced by different strains[a]

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
Sophorolipids				
Candida batistae	Diacetylated	75% ω-hydroxy fatty acids (etherified); mostly C18:1 acidic form, small content of C18:0 and C18:2	Glucose with olive oil	[136]
Candida floricola	Diacetylated	Acidic form	Glucose with olive oil (oleic acid)	[3, 180, 181]
Candida apicola	Mainly mono- and nonacetylated forms	Mainly consisting of lactonic, C18:,1 hydroxy fatty acid group is mainly ω-1 linked to the sophorose head group	Glucose with, oleic acid	[140] [141]
Candida riodocensis and Candida kuoi	Mainly diacetylated, small amount of mono- and nonacetylated	Mainly C18:1acid form, also lactonic ω-C18:1 form	Glucose with oleic acid	[140, 141]
Candida stellata	Mainly diacetylated, also mono- and nonacetylated	Mainly C18:1 acidic form	Glucose with oleic acid	[140]
Candida albicans O-13-1	Mainly diacetylated	Mainly C18:1 lactonic form, also C18:2 and C18:0	Glucose with sugar- cane molasses and soybean oil	[142],
Candida rugosa and C. tropicalis	Monoacetylated	Lactonic C18:1 or C20:4 acidic and lactonic form	Diesel oil	[182, 143]
Cyberlindnera samutprakarnensis	Suggestion: Non- and monoacetylated	Suggestion: C16:2 nonacetylated lactonic and C18:1 monoacetylated acidic form	Glucose with palm oil	[144, 150]
Lachancea thermotolerans		Acidic and lactonic form	Crude oil	[145]
Starmerella. Bombicola	Mainly diacetylated	Mainly C18:1 lactonic form, sometimes also C18:0 and C16:1 or C16:1 hydroxy fatty acid chain	Glucose or oleic acid	[140, 183]
Rhodotorula mucilaginosa	Diacetylated	C18:1 acidic form	Diesel oil	[182]

(continued)

Table 3 (continued)

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
Pseudohyphozyma bogoriensis	Mono- and diacetylated	13-hydroxydocosanoic acid (13-OH-C22)	Glucose	[71, 72]
Cryptococcus sp. VITGBN2	Diacetylated	C18:1 acidic form	Glucose and vegetable oil	[154]
Mannosylerythritol lipid				
Dirkmeia churashimaensis	MEL-A	C2, C4, C6, C14, C16	Glucose	[168]
Moesziomyces antarcticus	MEL-A,B,C, D	C8, C10	n-alkanes	[184]
		C8, C10, C10:1 C12, C14	Different vegetable oil (soybean, safflower, coconut, cottonseed, corn, palm oil)	[184, 185]
		Monoacylated, C8, C10, C12, C14	Glucose	[10]
		Triacylated, C8, C10, C18	Soybean oil	[186]
		C8, C10, C10:1 C12, C12:1,C14, C14:2, C16:0	Sucrose	[12]
		C8, C10, C10:1 C12, C12:1,C14, C14:2, C16:0	Olive oil	
Moesziomyces parantarcticus	MEL-A,B,C	C8, C10, C10:1	Soybean oil	[174, 172]
		Monoacylated, C8, C10, C12, C14	Glucose	[10]
		Triacylated, C8, C10, C18	Soybean oil	[172]
	MRL mannosylribitol lipid	C8, C10	Olive oil, ribitol	[17]
	MAL mannosyl-D-arabitol lipid	C8, C10	Olive oil, D-arabitol	[17]
	MML mannosyl-D-mannitol lipid	MML: C8, C10, C10:1, C12	Olive oil, D-mannitol	[16]
	MEL-A, MML-A	Diacylated C12/C12 and C10/C8	Different feed (soybean, rapeseed, olive, coconut oil)	[187]
Moesziomyces rugulosus	MEL-A,B,C	C8, C8:1, C10, C10:1, C10:2, C12:1, C13, C14:2	Soybean oil	[173]

(continued)

Table 3 (continued)

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
		Triacylated, C8,C10, C18	Soybean oil	[186]
Pseudozyma tsukubaensis pro tem.	MEL-B diastereomer	C8, C12, C14	Olive oil	[175]
	MEL-B	C6, C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C12:2, C14, C12:1, C14:2	Olive oil	[164]
	MEL-B	With additional OH at C14:	Castor oil	[18]
	MLAL mannosyl-L-arabitol lipid	C8, C12, C14	Soybean oil, L-arabitol	[19]
	MEL-A,B,C	Diacylated C14:1/C8:1 and C12:1/C8:0	Different feed (soybean, rapeseed, olive, coconut oil)	[187]
Sporisorium sp. aff. Sorghi	MEL-A	C12, C14, C16 or C14:1	Soybean oil	[188]
Triodiomyces crassus	MEL-A,B,C diastereomer	C2, C4, C14, C16, C18	Glucose and oleic acid	[15]
Ustilago cynodontis	MEL-C	C2,C4, C6, C14, C14:2, C16, C16:1	Soybean oil	[169]
Ustilago shanxiensis	MEL-C	C2, C4, C14, C16	Soybean oil	[170]
	MEL-B,C,D	Diacylated C16:0/C4:0 and C16:2/C2:0	Soybean oil, further oils are tested (rapeseed, olive, coconut oil)	[187]
Ustilago siamensis	MEL-B,C	C2, C4, C14, C16	Safflower oil	[25]
	MEL-A,B,C	C12:1, C14, C16, C16:1, C18, C18:1 C18:2	Sucrose	[12]
		C14,C14:1, C14:2, C16, C16:1, C16:2, C18:2	Olive oil	
	MEL-A,B,C,D MML-A, MML-B/C	Diacylated for MEL-B C16:0/C4:0 and 16:2/C2:0; for MML-B/C C16:0/C4:0	Different feed (soybean, rapeseed, olive, coconut oil)	[187]
Moesziomyces aphidis[b]	MEL-A,B,C,D; low amounts CL	C8, C10, C12	Soybean oil and glucose	[13]

(continued)

Table 3 (continued)

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
	MELs	C8, C10, C10:1, C10:2, C12, C12:1, C14, C14:1, C14:2, C16, C16:1	Glucose	[189]
	MELs	C8, C8:1, C10, C10:1, C10:2, C12, C12:1, C14:2	Soybean oil	
	MEL-A,B,C,D; MML-A, MML-B/C	Diacylated for MEL-A C10:1/C10:0 and C10:0/C8:0; for MEL-B and MEL-C C10:1/10:0	Different feed (soybean, rapeseed, olive, coconut oil)	[187]
	CL-B and MEL	Main CL-B: Two further hydroxy-groups in C16 acid part and acylated hydroxy-C6 acid at C2"	Glucose and soybean under nitrogen-limitation	[157, 190]
Mannosylerythritol lipid and cellobiose lipids				
Kalmanozyma fusiformata	MEL, CL	C8, C10, C10:1, C12	Soybean oil	[174]
	CL-B	CL: Further two hydroxyl groups in C16 acid part and acylated hydroxy-C6 acid at C2"	Glucose	[191]
Pseudozyma hubeiensis pro tem.	MEL-A,C; low amounts CL	C6, C10, C12, C16	Olive oil and glucose	[22]
	MEL-A,B,C; MML-A; MML-B/C	Diacylated for MEL-A and MEL-B C12:0/C6:0	Different feed (soybean, rapeseed, olive, coconut oil)	[187]
	CL-B and MEL	CL: Two further hydroxyl-groups in the C16 acid part and acylated hydroxy-C6 acid at C2"	Glucose, soybean oil, nitrogen-limitation	[190]
	MEL-C	C8, C10, C12, C14:2, C14:3, C16, C16:1, C18:2	Soybean oil	[23]
Sporisorium graminicola	MEL-A,B,C; low amounts CL	C6, C8, C12, C14; C14:1	Soybean oil	[21]
	MEL-A,B,C,D	Diacylated for MEL-A and MEL-C C14:0/C8:0 and C14:0/C6:0; for MEL-B C14:0/C6:0	Different feed (soybean, rapeseed, olive, coconut oil)	[187]

(continued)

Table 3 (continued)

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
	CL-B and MEL	CL: Two further hydroxyl-groups in C16 acid part and acylated hydroxy-C6 acid at C2" and acetylation at C6"	Glucose	[192]
	CL and MEL	CL: Two further hydroxyl-groups in C16 acid part and acylated hydroxy-C6 acid at C2"	Glucose, soybean oil, nitrogen-limitation	[190]
Sporisorium scitamineum	MEL-A,B; low amounts CL	C8, C10, C12, C14	Sugar cane	[20]
	MEL-A, B, C	C6,C8, C10, C10:1, C12, C12:1, C12:2, C14, C14:1	Sucrose	[12]
		C8, C10, C10:1 C12	Olive oil	
Ustilago maydis	MEL-A,B and CL-A.B,C	MEL mainly C6 and C14:1 CL: Two further hydroxyl-groups in C16 fatty acid; hydroxy-C8 at C2", small amount deacetylated CL-A	Glucose or sunflower oil	[157]
	CL	Ustilaginic acid, one or two further hydroxyl groups in C16 acid part, acylated with hydroxyl-C6 at C2", acetylated at C6'	Glucose, nitrogen starvation	[193]
	MEL-A, B, C	C6,C8, C10, C12, C14, C14:1, C14:2, C16, C16:1, C16:2, C18:1, C18:2	Sucrose	[12]
		C8, C10, C10:1 C12	Olive oil	
Cellobiose lipids				
Anthracocystis flocculosa	Flocculosin, CL with an additional acetyl group	One further hydroxyl-groups at C16 acid part; acylated hydroxy-C6 acid at C2"	Sucrose	[194]
Cryptococcus humicola	CL; with four to five acetyl groups at the sugar group	One or two further hydroxyl-groups at C16 acid part	Glucose	[26, 27]

(continued)

Table 3 (continued)

Species	Acetylation of sugar	Most common fatty acid residue	Carbon source	References
Trichosporon porosum	CL; with four to five acetyl groups at the sugar group	One or two further hydroxyl-groups at C16 acid part	Malt	[162]
Sympodiomycopsis paphiopedili	CL	Two further hydroxyl-groups at C16 acid part	Glucose	[158]

[a]A clear distinction of which strains make exclusively MEL, CL, or a mixture is only possible to a limited extent. The subdivision was made by the literature references of the measurements used. There should certainly still be investigations at the genomic level in order to find the gene clusters for the metabolic pathways or to prove the production of the respective biosurfactants in the laboratory. Most abundant variant is underlined

[b]Guenther et al. showed for *M. aphidis* DSM70725 that not all CL cluster genes homologs ORFs for the CL pathway are found. These results indicate that a CL cluster as observed in *U. maydis* does not exist in *M. aphidis* [195]

fatty acid with a chain length of usually C16, resulting in different CL structure variants. Depending on the producing strain, up to four side groups may be attached to the cellobiose molecule via ester bonds. CL-B is the main variant found in the produced CL mixtures of *U. maydis*, with up to two additional hydroxy-groups in the C16 acid and a (hydroxy)-hexanoic acid or (hydroxy)-octanoic acid residue at C2"position of the cellobiose; there is an aceylation group at C6'. CL-A is the deacylated structure and CL-C is an acylated form with an additional esterified carboxylic acid group [157, 193] (Fig. 1). CLs produced by *U. maydis* are also referred to as ustilagic acid and the CLs from *Anthracocystis flocculosa* (initially referred to as *Pseudozyma flocculosa*) are referred to as flocculosin, which is a CL structure with a further hydroxy-group in the C16 acid part, a hydroxylated C8 fatty acid chain esterified at C2" position and acetylation at C6' as well as at C3" [159]. Table 3 offers an overview of CL producing strains and the associated structures.

2.3 Metabolism and Genetic Engineering of Fungi

The metabolic pathways for GL produced by fungi and their genetic basis are not yet fully understood. Here we present the status of these pathways that are based on observations in laboratory experiments and comparisons between related microorganisms.

Van Bogaert et al. proposed the pathway of SL production using their own findings and combining published knowledge [197] (Fig. 3). In the first step, fatty acid is oxidized by a NADPH-dependent monochromoxydase enzyme cytrochrome

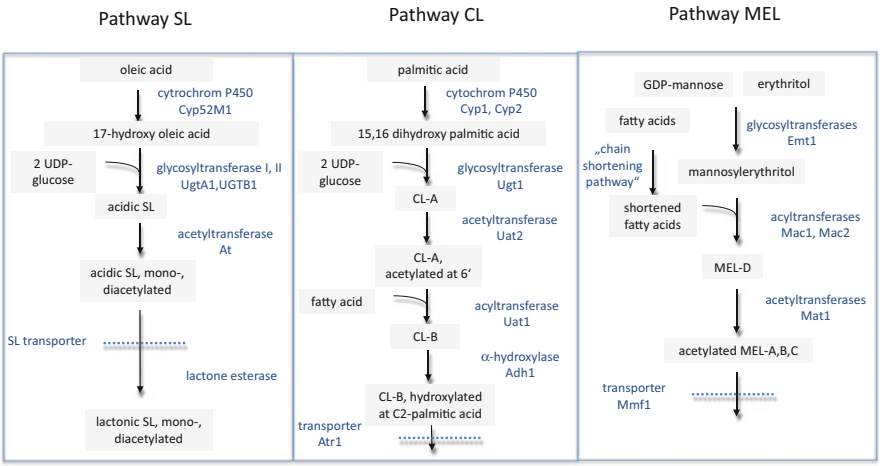


Fig. 3 Schematic models of the biosynthetic pathways for SL, CL, and MEL production

P450 to a hydroxyfatty acid, in terminal or subterminal positions. Then, glucose is glycosidically coupled to the hydroxyl-group of the fatty acid. SL is then acetylated by an acetyltransferase At. The conversion of the acidic SL is catalyzed by the enzyme lactone esterase. In 2007 a tool for genetic modification of *S. bombicola* ATCC 22214 was developed; the marker gene *ura3* was found [198], which allowed the development of a system for transformation and selection [199]. Thus, genes and enzymes could be identified that are involved in the SL biosynthesis: the enzyme glyceraldehyde-3-phosphate dehydrogenase [199], the gene *mfe-2* involved in β-oxidation [200] and three cytochrome P450 monooxygenases [201, 202]. Hence, the SL gene cluster was reported [203]. In another approach, the fatty acid-synthase complex was specifically inhibited by the addition of cerulenin so that no *de-novo* fatty acid synthesis could occur; and the resulting products were evaluated. Similar findings were achieved by cultivating a strain that impaired in β-oxidation [204]. The development of genetic tools made it possible to limit the large SL spectrum produced by a strain to produce defined SLs [205–208]. These publications show that metabolic engineering can produce a variety of SL molecules.

MEL biosynthesis cluster was first described in *U. maydis* [209] but was later also genetically explored in *M. antarcticus* [210, 211], *P. hubeiensis pro tem.* [212], *P. tsukubaensis pro tem.* [213], and *M. aphidis* [214]. Inducible promoters for controlled expression of genes for MEL producers were identified for *M. antarcticus* [215] and *U. maydis* [216]. The cluster consists of five genes encoding for a glycosyltransferase (Emt1, erythritol-mannosyl-transferase), two acyltransferases (Mac1 and Mac2, mannosylerythritol-acyl-transferases), one acetyltransferase (Mat1, mannosylerythritol-acetyl-transferase), and one cellular exporter (Mmf1, mannosylerythritol-major-facilitator protein). MEL biosynthesis pathway was first proposed by [209, 217] (Fig. 3). When using oil as carbon source, the first step is the secretion of lipases, which cleave the triglycerides into fatty acids

and glycerol. The regulation of lipase activity is strain specific. While *M. aphidis* only releases lipases into the medium in the presence of triglycerides, *M. antarcticus* shows 50% of its activity even without a hydrophobic carbon source [189]. For *M. aphidis* it is shown that most MEL cluster genes are strongly induced when hydrophobic carbon sources are present [195]. Within the MEL pathway the fatty acids are coupled to coenzyme-A and will enter either mitochondrial ß-oxidation or the so-called chain-shortening pathway. Here a partial ß-oxidation pathway takes place, where the fatty acids are shortened and afterwards incorporated into the MEL molecule [218, 219]. Prior to this reaction, an activated mannose is linked to erythritol via the enzyme glycosyltransferase Emt1. Emt1 has a key role, because its deletion completely prevents MEL biosynthesis [209, 216, 220]. In the next step, two fatty acids are combined by the two acyltransferases Mac1 and Mac2 with C2' and C3' of mannose to form MEL-D. As the chain length of the two residues differs in some strains, it is assumed that these enzymes have different substrate specificity [209]. As the last step, the acetyl-Co-A dependent acetyltransferase Mat1 binds one or two acetyl groups to C4' and C6' position of mannose rendering MEL-A,B, or C. Finally, MELs are exported by Mmf1 transporter [209].

Development of *ura3* deletion strains of P. *tsukubaensis pro tem.* and *M. antarcticus* enabled selection and detailed genetic modification studies [221, 222]. For *U. maydis* and *M. antarcticus* it is shown that MEL can no longer be formed when *emt1, mac1,* or *mac2* are deleted [209, 220]. However, a deletion of *mac2* in *P. tsukubaensis pro tem.* caused the secretion of monoacylated MEL with one fatty acid in C2' position [223]. MEL-D was mainly produced when *mat1* was deleted in *U. maydis* and *P. hubeiensis pro tem.* [24, 209]. In addition, the overexpression of lipase-encoding genes from *M. antarcticus* in *P. tsukubaensis pro tem.* resulted in an increased production of the diastereomer-type MEL-B [221]. In summary, it can be said that it will also be possible to produce tailor-made MEL molecules in the future.

U. maydis is the first and best described microbial producer of CL [224]. In 2006, the fully sequenced and annotated genome of *U. maydis* was published as the first fungus of the family *Ustilaginaceae*. It comprises a total sequence of 20.5 Mb, divided into 23 chromosomes with 6,902 genes [225]. Teichmann et al. were able to identify the complete 58 kb gene cluster of the CL synthesis, which comprises 12 genes [193]. They were able to clarify the biosynthetic pathway (Fig. 3) by generating deletion mutants of these genes. As a precursor of the CL synthesis palmitic acid is synthesized *de-novo*. Then, palmitic acid is terminally and subterminally hydroxylated by the cytochrome 450 monooxygenases Cyp1 and Cyp2. Subsequently, the UDP-glucose dependent glycosyltransferase links two glucose molecules o-glycosidically with dihydroxypalmitic acid to form the basic structure of CL-A. The acetyltransferase Uat2 then acetylates the cellobiose at C6'. After that another fatty acid is attached to the C2" position by the acyltransferase Uat1 to build CL-B. In turn, the hydroxylase Ahd 1 catalyzes the α-hydroxylation of palmitic acid. The transporter Atr1 seems to be necessary for the export of CLs [193, 226, 227].

As described, *U. maydis* contains both pathways, for MEL and CL production. For the regulation of the CL pathway, the gene sequence of Rua1, a zinc finger protein was identified [226, 228]. A deletion of the gene did prevent CL synthesis but not MEL synthesis. Therefore, the two glycolipids appear to be regulated differently. There is evidence suggesting that the glutamate level represses the Rua1 activity, which in turn is regulated by cell nitrogen content. Furthermore, the regulator Nit1 was also found outside the cluster, which can also regulate Rua1. Turning *nit1* off inhibits both MEL and CL synthesis. However, the exact regulatory mechanism has not yet been clarified [228]. In 2011, Teichmann et al. sequenced the genome of *A. flocculosa* and explored the gene cluster for the synthesis of flocculosin [226, 227]. Most of the genes in this cluster were homologous to *U. maydis* genes, however the cluster in *A. flocculosa* contains Fat3, an additional acetylase, presumably responsible for linking the additional acetyl group to C3".

2.4 GL Produced by Fungi in a Bioreactor

There are many publications available in literature reporting the growth of strains in small microtiter plates or shaking flasks. At this small scale, effects of different carbon sources, nitrogen sources, and media components on molecular structure, growth and production have been studied. However, if one moves toward industrial use, scale over into bioreactors and optimization of a purification method becomes necessary. The most common is the use of a stirred bioreactor combined with a multistep purification method including, e.g., filtration, solvent extraction, precipitation, or solubility changes due to pH and chromatography. All the described GL biosurfactant producing strains are aerobic and produce GL under aerobic conditions. The formation of biosurfactants is an oxidative process and requires oxygen molecules for their synthesis. Therefore, one of the major challenges facing GL production in bioreactors is the handling of the foam that is generated due to aeration of the bioreactor, intensified by the simultaneous production of GL biosurfactants. Controlled addition of oil can be used to reduce foam formation [111]. This is of great advantage since vegetable oils are already used alongside glucose as substrates for SL and MEL production. However, if the strains secrete lipases, for example, which cleave the triglycerides extracellularly to fatty acids, which are also surface active, the foaming may even be enhanced.

Most commonly, a GL fermentation process of those fungi starts with the growth stage toward biomass production and switches to the second stage in which a trigger initiates the GL production, as SL, MEL, and CL are part of the secondary metabolism [229]. However, in a batch process, biosurfactant formation could start in parallel with biomass production, when also the hydrophobic substrate is in the media [111]. It is worth noting that growth is a very energy intensive and an oxygen consuming process and could be separated from GL production. It would be desirable that as oxygen demand decreases during the GL production phase, growth slows or stops. A possible strategy is to derivate regulation of oxygen transfer to the

bioreactor, as this might help to better control the foaming. As a result, if aeration and agitation of the fermenter are reduced during GL production phase, less foam will be produced.

SL and MEL are produced using glucose and vegetable oils, feeding first and second generation substrates and waste streams, respectively. This is presented in recent excellent review articles [150, 177, 230, 231]. For most wild-type strains producing SLs and MELs, hydrophobic carbon sources such as plant oils, fatty acids, or alkanes are usually required for their production. In comparison, but in the case of CL production, no hydrophobic sources are required, only a sugar source [27, 114, 115]. An excellent overview for CL is given in recent reviews [232, 233].

For MELs the expression of this GL gene cluster at high level occurs especially at nitrogen starvation conditions [195]. CL production is also enhanced under nitrogen starvation [157, 169, 193]. SLs production starts at high amounts when nitrogen or phosphorous sources are depleted [234, 235].

Fungal GL production in bioreactors has been reported using different process strategies such as batch, repeated batch, fed batch with sequential or constant feeding or also few continuous processes in which an in situ product separation was aimed.

For SL there are several activities for production in bioreactors (Table 2 contains highlights for fed batch processes). *Starmerella bombicola* ATCC22214 is the most studied strain in fermentation processes. The highest titers were 300 g/L [104] using soybean oil and 422 g/L [107] using rapeseed oil as hydrophobic substrate within a fed batch process. With *S. bombicola* NRRL Y17069 120 g/L were achieved with sunflower oil [109]. For *Pseudohyphozyma bogoriensis* ATCC18809 the production of 51 g/L C22-SL was shown with glucose as substrate [71]. For MEL and CL, there are only a few publications in a stirred reactor (Table 2). With *Candida sp. SY16* (*P. tsukubaensis*) a titer of up to 95 g/L MEL in a 5-L reactor with soybean oil and glucose as carbon source was reported [110, 113]. *M. aphidis* DSM14930 produced up to 165 g/L MEL with soybean oil and glucose in a 72-L fermenter [111], while *M. aphidis* DSM70725 produced 70 g/L MEL [111, 112]. In turn, S. *scitamineum* NBRC32730 produced 25 g/L of MEL formation with sugarcane syrup in a 1.5-L reactor [20]. Up to 33 g/L CL was produced with different U.maydis strains in a fermenter [114–118], whereas with *C. humicola* JCM1461 13 g/L CL was reached in a 5-L reactor using glucose as feedstock [27].

However, we would like to point out that in some of the cases mentioned above a verification of the reported concentration should be carried out. The reason for this is that GL analysis methods have improved over the last few years and it was known until recently that MEL and CL can be produced simultaneously. It is often unclear, due to the production of the analytical preparations, whether a separation of CL and MEL or remaining oil or fatty acids from GL was achieved.

2.5 Downstream Processing of Fungal GL

After fermentation, GL needs to be separated from the aqueous broth containing inorganic salts, biomass, remaining fatty acids, oils, or sugars. SLs, MELs, and CLs

are usually secreted, so no cell disruption is necessary. Nevertheless, downstream processing is usually a multistep process with differently interconnected process units. Depending on the purification grade quality and necessary apparatus, this is a costly driver of the production process [236–238]. Separation becomes a major challenge due to surface-active properties of GL in the presence of microorganisms' surface components and remaining hydrophobic substrates (oil, fatty acids) that form a conglomerate or (micro)-emulsions [239, 240]. This mixture can only be separated by the combination of mechanical force such as centrifugation and (organic) solvents, salts, or agents. This then leads to a change in the solubility properties or physics of the surface of the substances within the conglomerate [238, 241]. Salting out effects are common in the petroleum industry for the de-emulsification of oil-water systems formed by enhanced oil recovery techniques [242].

For purification of GL, numerous publications are available for analytical purposes to follow the produced biosurfactant and the (hydrophobic) carbon source during the fermentation process. However, the development of simple scalable processes for downstream processing of GL that can be applied at an industrial scale would be a very valuable contribution to the field. Most of the published methods are based on the extraction of an organic solvent. For SLs, ethyl acetate is described as extraction solvent and the use of hexane for washing out the remaining hydrophobic substrate [109, 243, 244]. The extracted raw SLs could be purified by chromatography [245, 246]. To separate the lactonic and acidic SL forms, different methods such as precipitation and solvent extraction [247], the use of different adsorbents and elution solvents [248] and even crystallization have been described [142]. Examples of integrated approaches in which fermentation and separation of SLs could be implemented in parallel have been reported using a conical-bottom bioreactor with an integrated sieve plate [249, 250], or a settling column connected to the fermenter [251]. In another approach, SL could be separated due to their higher density compared to the fermentation liquid and a filtration setup was connected to the fermenter [150].

MEL extraction from cultivation broth is done by prior acidification to pH 2 or 3 [13, 111, 118, 171], or even without acidification using ethyl acetate as organic solvent (reference: nearly all literature deals with MEL cultivation). Acidification could lead to a chemical deacetylation [118], however, it could be used as a post-modification method as well as using lipases [252]. As an interesting preliminary stage, heating of the fermentation broth up to 110°C and cooling down led to separation of MEL and other hydrophobic compounds from the water phase and this makes the decantation possible [237] . In the so-called MEL, raw extract (from decantation as well as from solvent extraction) and free fatty acids or acylglycerides are contained as residues from broth cultivation. A higher-order multistep extraction with methanol, cyclohexane, and hexane was successful but had a low yield [237]. In contrast, chromatographic purification showed a high yield and high performance in purification of MEL [19, 23, 24, 187, 188, 253, 254]. Chromatography allows also separating the different MEL-A, -B, -C, -D variants [112]. Additionally, methods such as adsorption on XAD resins [237] or activated charcoal [255] followed by solvent elution with, e.g., MTBE or methanol are also described. A foam fractioning

could be integrated in an in situ recovery during the fermentation. Andrade et al. described combined micro- and ultrafiltration (100 kDa cut off) of a foam overflow in a fermentation process [239].

Roxbourg et al. first described CL production in a fermentation process using *U. maydis* and glucose as the only carbon source. CL was secreted and transformed in insoluble crystals in the cultivation broth and centrifuged after cultivation. Then CL was extracted with methanol and recrystallized. Contaminants like lipids were removed with ether [114]. Günther et al. showed that production of CL and parallel crystallization could be intensified at low pH [117, 118]. After fermentation, purification was performed by sedimentation of CL crystals at acidic pH, ethanol extraction, and washing of the recrystallized product using ethyl acetate. A similar method was used for the separation of flocculosin, in which cell suspension was acidified and filtered and flocculosin was extracted with chloroform [256]. It has been reported that for strains producing MEL and CL in parallel, extraction methods with MTBE, chloroform [115] or ethyl acetate with 2-propanol [190] can be used. Subsequently, MEL and CL could be separated from each other by chromatography [157, 190].

2.6 *Physical Properties, Biological Activity, and Application Potential of Fungal GLs*

One of the greatest successes in bringing GL biosurfactants produced by fungi to the market is SLs, produced by *Starmerella bombicola*. Fermentation processes at research level showed titers of 422 g/L [257]. MELs have also been reported to be used in commercialized products [258]. Here, especially the diastereomer type of MEL-B produced by *P. tsukubaensis pro tem.* is commercialized as a cosmetic ingredient [230]. Fermentation processes produced in research have reached titers up to 165 g/L, depending on strain and process conditions, which were achieved with *Moesziomyces aphidis* [111]. The highest titer reported for *Ustilago maydis* for the production of CLs was 33 g/L [114, 118]. However, CLs are not yet used commercially.

Inspired by the critical discussion in the reviews by Roelants et al., Claus and van Bogaert, and Irorere et al. [149, 150, 259], the authors would like to mention that these high titers should be taken with caution. This is because the quantification methods used until the early 2000s could lead to over-determination of these GLs. Having a deeper look into literature, the following sections will focus on the production of SL, MEL, and CL.

SLs, MELs, and CLs can present different structure, depending on the strain, feedstock, and the production process. In addition, the degree of purity and stability of the biosurfactant is important for the evaluation of the applications. For example, chemical changes of the molecules like deacetylation or chain length of the fatty acid residues can lead to changes in their properties. The next sections will give an

overview of the physical properties, like surface tension, CMC (also listed in Table 1), and biological activities, like antimicrobial, fungal activity, influences to cell lines.

2.6.1 SLs

An enormous number of publications are available for SLs; however, only selected ones are presented in this review. Although many SL properties are changed by varying their structure, the ability to lower the surface tension to an average value of 35 mN/m remains. This is true for most structural variants, when only the chain length of the main fatty acid is slightly varied or non- or diacetylated SLs were studied (compare this in Table 1). However, cleaving one glucose molecule from the sophorose increases the surface tension to 47.3 mN/m [3] and diacylation at C6' and C6" with a hydroxy-C10 group decreases surface tension to 27 mN/m [6]. Interestingly, different CMC values were measured: the greatest differences become apparent when SL is present in the lactonic form with 45.1 mg/L or acidic nonacetylated form with 245 mg/L [4]. SLs have self-assembly properties and can aggregate to supramolecules. Baccili et al. described this well in different publications [260–265]. For SLs, different biological activities were analyzed. Natural SLs from *S. bombicola* ATCC 22215 showed antimicrobial activity against *Cupriavidus necator* and *B. subtilis* and SLs were also able to disrupt biofilms (mixtures of *B. subtilis* and *Staphylococcus aureus*) [266]. SLs from the same strain (90% nonacetylated C18:1; 4 g/L) showed effective reduced growth of *Enterococcus faecalis* and *Pseudomonas aeruginosa*, whereupon the application is seen to be used as a wound healing agent [267]. Further studies on the pharmacological effect of SLs were performed: Different SLs (natural mixture, lactonic, acidic form, methyl ester, hexyl ester, mono-acetate ethyl ester, di-acetate ethyl ester) from *S. bombicola* ATCC 22214 were tested for antiviral (anti-HIV) and sperm immobilizing properties. Diacetate ethyl ester SL gave the best results, however, sensitivity to vaginal epithelial cell was also noted [268]. These SLs were also applied to human pancreatic cancer cells and produced promising results [269, 270]. Additionally, SLs showed good properties against sepsis [271]. Natural produced SLs from the same strain cause significant neurite outgrowth in PC12 cells [272]. These SLs also induced the human promyelocytic leukemia cell line HL60 to differentiate to monocytes, the human myelogenous leukemia cell line K562 and the human basophilic leukemia cell line KU812 to differentiate into monocytes, granulocytes, and megakaryocytes [273]. For SLs also immune modulatory effects are known; IgE production in U266 cells was decreased [274, 275]. For deeper insights, we recommend the original literature or several reviews [1, 276].

SLs are manufactured by various companies and can be used in a wide range of applications, such as in the cleaning and cosmetic industry or as additives in the food, pharmaceutical, and agricultural sectors. An overview of potential applications are given in an excellent review [150]. For an environmentally friendly use of SLs, complete biodegradability is an important aspect. This has been investigated for the

production of SLs from *S. bombicola* 22214, furthermore, no significant impact on the reproduction of *Daphnia magna* (water flea) was shown [277].

2.6.2 MELs

Different MEL variants can reduce the surface tension of water to a value between 24.2 and 30.8 mN/m. The CMC (critical micelle formation concentration) is for MEL-A-D usually between 2.6 and 15.8 $\times$ 10^{-6} mol/L. Rare MEL, like MML, MRL, MAL, was in the same range, monoacylated MEL showed surface tension decrease to 33.8 mN/m and a CMC of 3.6 $\times$ 10^{-4} mol/L. Most MELs were purified to such extent that only the individual variants MEL-A, B or C were obtained and measured. Synergistic effects from the determination of the naturally occurring MEL mixture are thus not recorded. An overview of the surface tension and CMS is given in Table 1. After exceeding the CMC, all MEL variants form three-dimensional, liquid-crystalline structures (lyotropic mesophases) by self-aggregation in aqueous solution and could aggregate to the so-called sponge, bi-continuous, and lamellar phases, resulting in formation of liposomes, vesicles, single-layer or multi-layer structures [278–280]. The number of acetyl groups in the MELs influences self-aggregation. With increasing concentration, MEL-B, -C, and -D form predominantly lamellar phases over a wide concentration range [280, 281]. In contrast, MEL-A changes the phase composition between sponge, lamellar, and bi-continuous cubic phase [278, 279]. Thermostable vesicles can be generated by dispersion of the sponge phase and are suitable for transport and delivery of active substances [282], or gene transfer into human cells [283, 284]. For MEL-A without addition of salts or co-surfactants the formation of W/O microemulsions was detected. With MEL-B, O/LC-emulsions (oil in liquid-crystalline phase) could be produced, whereby a stable gel was formed [285]. Different applications could be shown for these properties. Self-aggregated monolayers of MEL-A from *M. antarcticus* T-34 coated to poly (2-hydroxyethyl methacrylate) (pHEMA) beads bind human immunoglobulin IgG with high affinity and could be used as ligand for affinity chromatography [286]. Imura et al. showed a high affinity of self-assembled monolayers of MEL-A to IGM and IgM antibodies [287]. Inoh et al. indicate that gene transfection into mammalian cultured cells could be efficient by using liposomes containing MEL-A [283, 284]. More insights on their three-dimensional behavior are also given in the several review articles [281, 288].

In addition to these physical-chemical properties, MELs have different biochemical effects. Most of the MEL-A and MEL-B investigated were synthesized with *M. antarcticus* T-34. Both, MEL-A and B had an effect against gram-positive bacteria, like *Bacillus subtilis, Micrococcus luteus, Mycobacterium rhodochrous, Staphylococcus aureus,* and *Pseudomonas rivoflavina* [7]. For monoacylated MEL a less antimicrobial activity was investigated [10]. Interestingly, there is no publication about antifungal activity.

Effects on cell lines were also investigated: MELs from *M. aphidis* DSM 70725 induced melanoma cell apoptosis [14]. For MEL-A from *M. antarcticus* T-34 it was

found that PC12 cells had a significant neurite outgrowth [272]. MEL-A and B from the same strain induced malignant melanoma B16 cell lines at 5 μM, but caused apoptosis at >10 μM [8, 289]. These MELs also induced the differentiation of the human myelogenous leukemia cell line K562 [290], the human basophilic leukemia cell line KU812 into monocytes, granulocytes, and megakaryocytes [291] and the differentiation of human promyelocytic leukemia cell line HL60 into granulocytes. Furthermore, it was observed that these MELs inhibited the activity of phospholipid- and Ca^{2+}-dependent protein kinase [273, 291]. For MELs derived from *M. antarcticus* KCTC7804 the toxicity of mouse fibroblast L929 with $LD_{50} = 5$ g/L was measured. MELs from the same strain also proved to be readily biodegradable [11].

For a possible application, the cosmetics sector was suggested, as MELs from *M. antarcticus* T-34 and *P. tsukubaensis pro tem.* NBRC 1940 have a strong rehydrating and regenerating effect on skin and hair. This effect is known from ceramides [292–294]. Furthermore, MELs from *M. antarcticus* T-34 could be used as an anti-agglomeration agent to suppress agglomeration and growth of ice-particles [185]. MELs from the same strain could also be used as additives for plant protection due to their wettability on hydrophobic surfaces [295, 296].

2.6.3 CLs

For CLs, only few data for surface tension and CMC value are available, an overview is given in Table 1. However, many studies on antifungal and antimicrobial effects have been conducted for CLs. One of the oldest studies was conducted in 1951 for *U. zeae* PRL 112 (later referred to as *U. maydis*) [297]. Haskin and Thorn tested 10 bacteria and 27 fungi. The following gram-positive and -negative bacteria showed the best effects: *Micrococcus pyogenes var. aureus, Xanthomonas camestris, X. translucens cerealis, Bacillus subtilis, Corynebacterium flaccumfaciens, Brucella bronchiseptica, Mycobacterium butyricum;* and the fungi: *Thielaviopsis basicola, Streptomyces griseus, Ascochyta sp., Claviceps purpurea* and *Neurospora sitophila.*[2] In addition, preliminary tests with rats and white mice have shown that CL is relatively non-toxic when administered by feeding or intraperitoneal injections [297]. Later, several other strains were discovered and analyzed. For *K. fusiformata* VKM Y-2821 and *C. humicola* 9–6 an antifungal activity against *Filobasidiella neoformans*, *C. terreus, S. cerevisiae, C. albicans, C. glabrata. C. viswanathii,* and *Sclerotinia sclerotiorum* was shown [298, 299]. Flocculosin from *A. flocculosa* was used to test ~65 bacteria strains and was found to have high activity against gram-positive and -negative bacteria, with the best activity shown against *Staphylococcus aureus* [300]. Good fungal activity was displayed against *C. glabrata, S. cerevisiae, C. krusei,* and *C. parapsilosis,* while activity against *C. albicans, C. lusitaniae, Cr. Neoformans,* and *Trichosporon asahii* was

[2]Original strain names from literature were used.

low [159, 300]. About 52 genera of fungi showed sensitivity to CLs from *Trichosporon porosum* strains, whereas a high sensitivity (cell death) was shown for C. *albicans, Filobasidiella neoformans, S. cerevisiae,* and *C. terreus* when using CLs from *T. porosium* VKM-Y-2056 [162]. CLs from *Sympodiomycopsis paphiopedili* VKM Y-2817 showed a good activity against *C. terreus, C. albicans* [158]. For CLs from *S. graminicola* L1-20, L1-46 about 377 fungal strains were tested and 95 genera, e.g. *C. albicans, C. glabrata, C. viswanathii, Clavispora lusitaniae, S. cerevisiae, C. terreus,* and *F. neoformans* were sensitive to them [192]. Possible applications for CLs would therefore be as a preservative in the food and animal feed sector or as a fungicide in agricultural sector [300]. It was found that the acetyl groups in the cellobiose molecule and the OH- groups in palmitic acid are essentially responsible for the fungicidal effect. It was assumed that these side groups protect against degrading fungal enzymes [301]. The antibiotic effect of CLs was shown by affecting the cell membrane, in which it led to leakage of potassium ions and ATP [161, 302].

For flocculosin from *A. flocculosa* their toxic effect on cell lines was also investigated. There was no effect on human cancer cell lines, like T24, Rupp2, Lovo, HepG2, HACAT, CHODOFF, and hemolysis of sheep erythrocytes [159].

CLs form needle-shaped crystal-structures under acidic conditions. Self-assembly properties were shown for CLs from *U. maydis* [264]. If the pH value rises above pH 7, they form a gel-like mass in aqueous solution. These gelling properties were investigated by Imura et al. [303]. It has been found that solid gels are formed in various solvents, including ethanol-butanediol-water mixtures. According to Imura et al., CLs thus have the potential to be used as low molecular weight gelators in hydrogels or organogels. These are currently used in cosmetics, for transport of active substances, in biosensors and for the processing of designed food [303].

3 Concluding Remarks

The industrial application of GL biosurfactants is now a reality, since both RL and SL containing products have reached the market. However, there are several points that need to be studied to explore the full potential of these fascinating molecules. There are ample research opportunities that include the isolation and characterization of novel bacteria and fungi that exhibit increased GL biosurfactant production to understand the metabolic pathways and genetic regulation circuits involved in the synthesis of these tension active compounds. This could lead to genetic manipulation of these microorganisms to enhance GL production. In addition, there are important challenges in the development of large-scale fermentation and downstream processes that make GL biosurfactants competitive to increase their share in the surfactant market.

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Bacillus sp.: A Remarkable Source of Bioactive Lipopeptides

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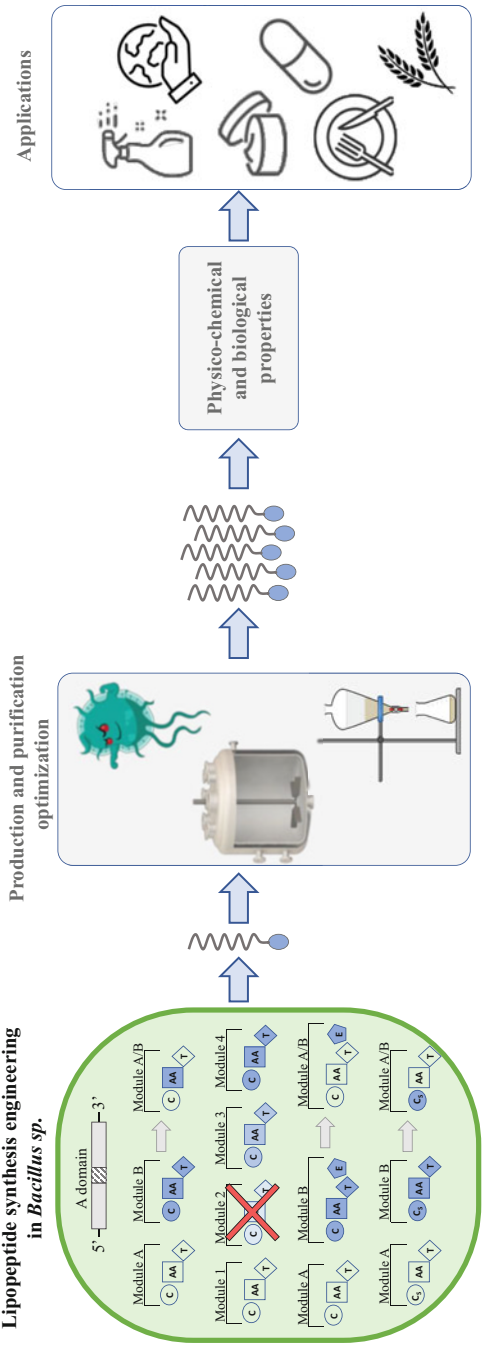
Abstract Surfactin, one of the best lipopeptide surfactants, was first isolated from *Bacillus* sp. in 1969. Since then, *Bacillus* sp. has been a remarkable source of bioactive lipopeptides, with a huge natural biodiversity. Lipopeptides from *Bacillus* sp. are now divided into three main families: surfactin, fengycin, and iturin. The peptide moiety of these lipopeptides is synthesised by huge multi-enzymatic proteins called nonribosomal peptide synthetases, which are responsible for the peptide biodiversity of these lipopeptides. Moreover, the fatty acid chain also encompasses a high diversity with different β-hydroxy or β-amino fatty acid chains of different lengths, isomery, or saturation, which can be incorporated. After describing the mode of synthesis of the different families of lipopeptides produced by *Bacillus* sp. and their biodiversity, this chapter describes how this lipopeptide biodiversity can be increased using genetic engineering and how the lipopeptides can be overproduced and purified. The high biodiversity of lipopeptides induces a broad range of physicochemical properties, which can be linked to multiple biological activities with many applications in different sectors. The increasing understanding of the mode of biosynthesis of these lipopeptides should lead to the development of novel compounds with increased properties and applications.

Graphical Abstract



Keywords *Bacillus*, Lipopeptide, NRPS, Genetic engineering, Biodiversity, Physicochemical properties, Bioactivity, Application

1 Lipopeptide Biosynthesis and Natural Biodiversity

1.1 Nonribosomal Peptide Synthesis

Lipopeptides produced by *Bacillus* strains are divided into three main families of compounds: surfactin, iturin, and fengycin [1], produced by different strains belonging to the *Bacillus subtilis* or *Bacillus cereus* groups and a set of molecules produced by one or a very limited number of strains, such as kurstakin [2], antiadhesin [3], bamylocin A [4], circulocin [5, 6], licheniformin [7], and locillomycin [8]. All of them are composed of β-hydroxy (surfactin, fengycin, kurstakin, antiadhesin, bamylocin A, circulocin, and locillomycin) or β-amino (iturin and licheniformin) fatty acid (FA) chain of different lengths, isomery, or saturation (fengycin). Guanylated fatty acid (gFA) chains have been detected in circulocins. The peptide moiety consists of five (circulocin 1), six (circulocin 3), seven (surfactin, iturin, antiadhesin, bamylocin, and licheniformin), nine (locillomycin), or ten (fengycin) amino acid residues of the L or D form. All lipopeptides are cyclised or partially cyclised; however, in some cases, linear forms are concomitantly observed [9]. All of them are synthesised by nonribosomal peptide synthetases (NRPS) with the help of polyketide synthases (PKS) in the case of iturin or locillomycin. These two types of bio-catalysers are multi-enzymatic proteins consisting of repeated modules, which function as assembly line machinery for the biosynthesis of a high set of bioactive microbial secondary metabolites [10]. The first NRPS complex was described by Lipmann et al. in 1971 [11] and is responsible for the biosynthesis of gramicidin S, an antibiotic produced by *B. subtilis*. Since this discovery, several thousands of compounds synthesised by this mechanism have been characterised. Most of them are gathered in a database called NORINE [12]. Interestingly, this mode of biosynthesis is mainly responsible for the high biodiversity observed in lipopeptide structures. Each NRPS is subdivided into modules, which contain the set of enzyme activities necessary to catalyse the incorporation of one specific amino acid into a peptide backbone. Five main catalytic activities, called domains, are used in NRPSs responsible for the biosynthesis of lipopeptides in *Bacillus* sp. The adenylation (A) domain recognises one amino acid residue and catalyses its transformation into amino-acyl adenylate by a reaction with ATP. Several A domains show low specificity and can activate amino acid residues with structural similarities, such as valine, leucine, and isoleucine. This low specificity induces biodiversity in lipopeptides synthesised by the same NRPS. The peptidyl carrier protein (PCP) domain, also called the thiolation (T) domain, has to be transformed from apo-protein in active holo-protein by the addition of a serine residue of a phosphopantetheine arm, which is a part of coenzyme A. This transformation is

catalysed by a phosphopantetheinyl transferase encoded in *Bacillus* by the sfp gene [13, 14]. It generates a sulfhydryl group that can react with an aminoacyl adenylate to create a thioester bond between the carboxylate group of the amino acid residue and the PCP domain. The third domain is the condensation (C) domain, which catalyses (1) the formation of a peptide bond between an acceptor substrate (the nascent peptide) and a donor substrate (the amino acid carried by the adjacent module) and allows for (2) the subsequent translocation of the growing chain to the following module. This C domain can be separated into two subdomains called C-donor (C_D) and C-acceptor (C_A), each of which is specific for the previous and current amino acids, respectively [15]. One main characteristic of NRPS responsible for the biosynthesis of lipopeptides is the presence of a C starter (C_S) domain that catalyses the acylation of the amino acid residue activated by the first module [15]. This C_S domain is known for its low specificity and ability to incorporate FA of different lengths and isomeries. Sometimes, an epimerisation (E) domain can be present in the module and will catalyse the conversion of the L-amino acid residue previously activated and fixed in the PCP domain of the module in a D-amino acid residue. The last domain, a thioesterase (TE), is necessary for the release of the peptide and its cyclisation. This domain is only present in the last module. It was not clear whether the presence of linear forms observed with surfactins, for example, is the result of either an inefficient activity of the thioesterase or the presence of a lactonase, which could open the cycle. In any case, this would increase the biodiversity of the compounds produced. For some synthetases of lipopeptides, a second thioesterase is present and is involved in the initiation of the synthesis. Except for locillomycin, NRPSs involved in lipopeptide synthesis operate according to a linear synthesis, with an initiation module (C_S-A-T) able to recognise the first amino acid residue, followed by as many modules (C-A-T) as monomers required to complete the peptide and a last module (C-A-T-TE), which will incorporate the last monomer, release, and cyclise the lipopeptide. As previously mentioned, PKS is involved in the synthesis of iturin and locillomycin lipopeptides. For iturin, three catalytic domains are responsible for the last steps of FA biosynthesis (last elongation and β-amination [16]) before its transfer to the first amino acid of the peptide moiety (acyl-CoA ligase (AL domain), acyl carrier protein (ACP domain), β-ketoacyl synthetase (KS-domain) and amino transferase (AMT domain)). Hansen et al. [17] have shown that the AL domain can activate free FA through an acyl-adenylate intermediate and load it on the adjacent T domain independently of coenzyme A. Compared to other lipopeptides, the biosynthesis of locillomycin is specific. It contains three catalytic domains of PKS, a fatty acid acyl-coenzyme A synthetase (ACS) domain, an acyl carrier protein/thiolation (T) domain, and a β-ketoacyl synthetase (KS) domain; however, this last domain seems to be skipped. On the NRPS side, a synthetase with three modules is iteratively used with an epimerase, which appears to function optionally.

1.2 Biodiversity of Surfactins

Surfactin is one of the three main lipopeptide families produced by *Bacillus* sp. All of the surfactins are heptapeptides, with the chiral sequence LLDLLDL, and have an FA linked to the peptide chain by lactone closure (Fig. 1).

For the surfactin family, four genes encode NRPS: *srfAA*, *srfAB*, *srfAC*, and *srfAD*. From the heptamodular NRPS obtained from these genes, *srfAA* codes for the first three modules, *srfAB* codes for the next three modules, *srfAC* codes for the seventh module and a thioesterase, and *srfAD* codes for a second thioesterase/acyltransferase domain that stimulates surfactin biosynthesis initiation (Fig. 2).

In this family, three main types of lipopeptides have been identified. Surfactin is produced by *Bacillus* sp., including *B. subtilis*, *B. amyloliquefaciens*, and *B. velezensis*, lichenysin is produced by *B. licheniformis* [18], and pumilacidin is produced by *B. pumilus* [19]. These differences are in the peptide chain and amino acid composition.

Surfactin is composed of Glu_1-Leu_2-Leu_3-Val_4-Asp_5-Leu_6-Leu_7, whereas lichenysin has a glutamine residue at position 1 instead of a glutamic acid residue, and pumilacidin has a leucine residue at position 4 instead of a valine residue and an isoleucine residue at position 7 instead of a leucine residue.

Furthermore, a different level of diversity was observed. For the same strain, there can be a change in one amino acid of the lipopeptide produced, and thus the production of different peptide chains. The origin of this change is the non-specificity of the A domain in NRPS. For positions 2, 4, and 7, there was greater variability and the amino acid accepted belonged to the aliphatic group (leucine, valine, isoleucine, and alanine) [20, 21]. For the other positions 1, 3, 5, and 6, the lack of variability could be due to the type of amino acid (negatively charged for Glu_1 and Asp_5) and epimerisation (D-Leu at positions 3 and 6). In addition to the peptide chain variation, there is also a variation in the length of the FA or its isomery. For the surfactin family, the chain can vary from 12 to 17 carbon atoms and have a linear (*n*) or branched configuration (*iso* and *anteiso*).

Lastly, surfactin forms that could result from a chemical change have nonetheless been observed naturally. Linear surfactins have been produced by *Bacillus* strains [9] and surfactin methyl esters have been produced by *B. subtilis* [22], *B. licheniformis* [23], and *B. pumilus* [24].

1.3 Biodiversity of Fengycins

Fengycins or plipastatins were described in 1986, isolated for the first time from *B. subtilis* and *B. cereus* strains, and then proved to be identical compounds [25]. Several studies have reported their production from other *Bacillus* species [26–28].

These molecules are lipodecapeptides and display an internal lactone ring in the peptide moiety between the carboxyl terminal amino acid (Ile) and the hydroxyl

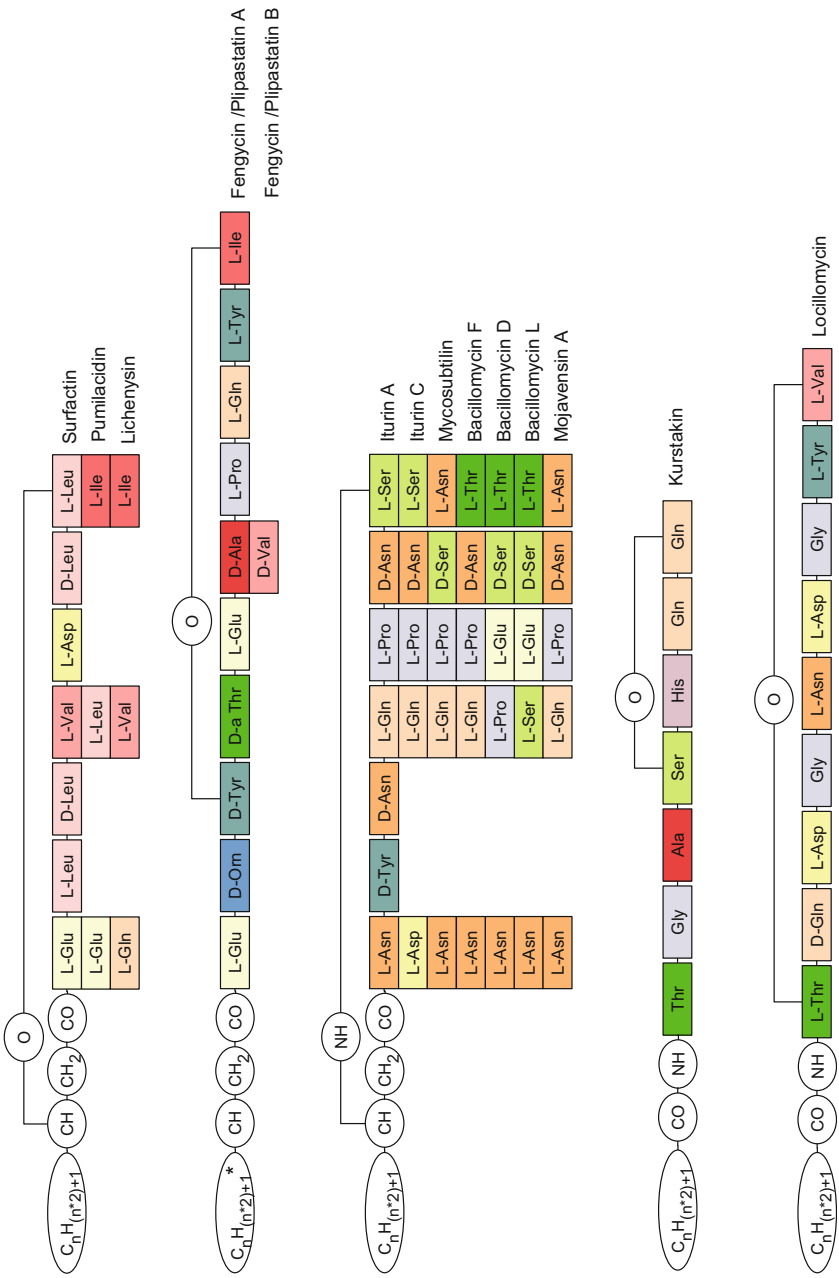


Fig. 1 Structure of the main forms of the lipopeptide families produced by *Bacillus* sp. and their variants (such as surfactin also exists with valine at position 7). Amino acid residues are written using the three-letter code. The L or D configuration is indicated before the amino acid residue. *For the fengycin family, the fatty acid chain can be unsaturated

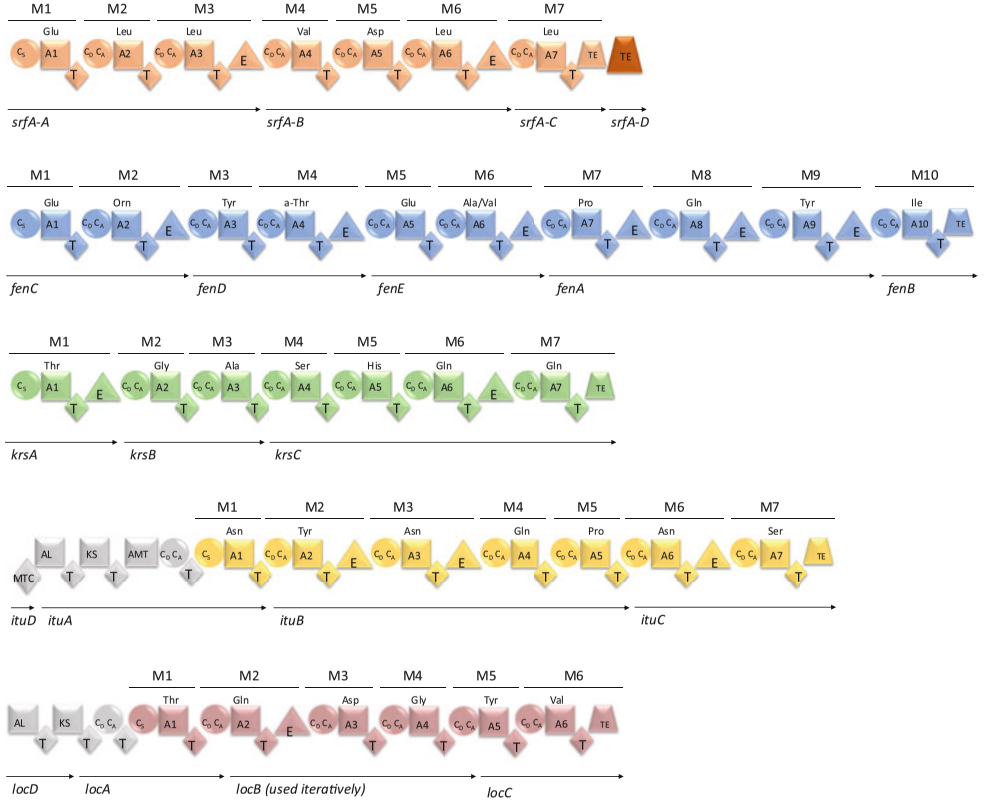


Fig. 2 Schematic representation of the gene clusters of surfactin (orange), fengycin (blue), kurstakin (green), iturin (grey and yellow), and locillomycin (grey and red). The gene clusters contain the ORFs and the domains corresponding to the nonribosomal peptide synthetase with the different modules (M) incorporating the amino acids. The polyketide synthases are represented in grey. *A* adenylation domain, C_S condensation starter domain, C_D condensation domain donor, C_A condensation domain acceptor, *T* thiolation domain, *E* epimerisation domain, *TE* thioesterase, *MTC* malonyl-CoA transacylase, *AL* acyl-CoA ligase, *KS* β-ketoacyl synthetase domain, *AMT* amino transferase

group in the side chain of the tyrosine residue at position 3 [29, 30] (Fig. 1). Fengycin or plipastatin NRPS is encoded by five genes: *ppsA*, *ppsB*, *ppsC*, *ppsD*, and *ppsE*, which correspond to *fenC*, *fenD*, *fenE*, *fenA*, and *fenB* [31] (Fig. 2). The first three enzymes contain two modules. For *fenC*: *fenC1* and *fenC2*, which activate Glu and Orn, respectively [32], *fenD*: *fenD1* and *fenD2* activate Tyr and Thr, respectively [33], and *fenE* with two modules: *fenE1* activates Glu and *fenE2,* which is less specific, can activate two different amino acids, Ala and Val. Val is activated in fengycin B, whereas Ala is incorporated into fengycin A [34]. The enzyme *fenA* contains three modules: *fenA1*, *fenA2*, and *fenA3*, which activate Pro, Gln, and Tyr, respectively [35]. Finally, *fenB* activates the last amino acid, Ile, and a thioesterase domain involved in the release of the peptide and the formation of an ester bond between the last and third amino acids of the peptide moiety [36].

To date, large structural heterogeneity has been reported in the fengycin family. This biodiversity is generated by variations in both the β-hydroxy fatty acid moiety and the partially cyclic amino acid chain. Fengycins A and B have been reported as the two main classes of fengycin, differing in their sixth amino acid of the peptide chain [30]. There are only two other variants reported in the literature that contain a different amino acid in that position: one structure of fengycin, including an aminobutyric acid (abu_6) [37, 38], and a second fengycin variant with Leu/Ile_6 and Ile_{10} [39]. Furthermore, several structures have been described and confirmed recently: fengycin A2 (Ala_6 and Val_{10}), fengycin B2 (Val_6 and Ala_{10}) [39], fengycin C (Ala_6 and Thr_9) [40], fengycin D (Val_6 and Val_{10}), and S (Ser_3, Val_6, and Ile_{10}) [38, 41], and, recently, fengycin X (Ala_6 and Leu/Ile_8), and Y (Val_6 and Leu/Ile_8) [42]. To date, all reported fengycins contain Glu residues at positions 1 and 5 and a Gln residue at position 8, except in one report in which the analysis showed a rare form of fengycin from *B. subtilis* K1 containing only Glu in the three positions [39].

The β-hydroxy FA moiety varies from C14 to C18, with C15, C16, and C17 being the main representative FA. The lipid moiety is linked by an amide bond to the N-terminal amino acid residue (Glu_1) [29, 30]. The most common forms are saturated, except in some cases where a C15 β-hydroxy FA includes a mono-unsaturation between carbon C13 and C14 [39, 43].

1.4 Biodiversity of Iturins

The iturin family was discovered in 1950 [44] and was defined as lipoheptapeptides interlinked with a β-amino FA of length varying from C14 to C17 [45] (Fig. 1). They are known to be strong antimicrobial compounds that are active against yeast and most fungal phytopathogens [46–48].

Iturins are synthesised by a PKS–NRPS hybrid complex [49]. The operon coding for iturins contains four open reading frames: *fenF*, *mycA*, *mycB*, and *mycC* for mycosubtilin [50] and *bmyD*, *bmyA*, *bmyB*, and *bmyC* for bacillomycin D [51]. The iturin A operon is synthesised by *ituD*, *ituA*, *ituB*, and *ituC* (Fig. 2). *ItuD* is essential, specifically for iturin A, as it encodes malonyl coenzyme A transferase, which participates in the formation of side FA. In addition, its deletion leads to loss of

production of iturin A [52]. The ituA gene encodes the PKS modules responsible for the last elongation, β-amination, and incorporation of the acyl chain, and the first amino acid residue (Asn). The ituB gene encodes an NRPS of four modules responsible for the incorporation of Tyr, Asn, Gln, and Pro. The last gene, *ituC*, encodes a two-module protein incorporating the last two amino acid residues (Asn and Ser) and releases the peptide. Two epimerisation domains in modules 3 and 6 transform L-Asn into D-Asn.

All of the compounds within the iturin family share some common structural characteristics: (a) the peptide cycle is characterised by a constant LDDLLDL chiral sequence of the amino acid residues; (b) the first three amino acids correspond to a conserved pattern (L-Asx_1 D-Tyr_2 D-Asn_3); (c) the last four amino acids constituting the peptide moiety are variable, depending on the compound [20]. In addition to the structural variations in the peptide moiety, the classification of each molecule also depends on the length of the FA [53].

Seven iturins have been reported to date, with iturin A being the most studied, and its complete structure was elucidated by Peypoux et al. in 1978 [45]. Iturin A is a heptapeptide containing L-Asn, D-Tyr, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser, related to a C14 or C15 fatty acyl chain [45]. Similar to iturin A, iturin C [54], bacillomycin D [55], and bacillomycin L [56] (which is the same as bacillomycin Lc [57, 58]), all exhibited the same length of the lipid moiety. In contrast, bacillomycin F [59] and mycosubtilin [60] have long C16 and C17 forms, similar to iturin A_L [47]. More recently, a new iturinic lipopeptide, mojavensin A, was isolated from *Bacillus mojavensis* B0621A [61].

1.5 Other Lipopeptides from Bacillus *sp.*

Numerous other lipopeptides were isolated, and their structures and biosynthesis characterised from *Bacillus* sp. These metabolites are all composed of either an FA or a gFA, linked by an amide or ester bond to a cyclic or partially cyclic peptide chain. The latter contains 5 to 13 amino acids, some of which appear in the D-form.

In *Bacillus* sp., these lipopeptides include the following:

1.5.1 Antiadhesin

Antiadhesin from *B. licheniformis* 603 is an FA-O-heptapeptide with antiadhesive activity [3]. This lipopeptide consists of a heptapeptide, N-acylated to the N-terminal amino acid, L-Asp, by a 3-hydroxy FA (from 13:0 to 17:0 with *n*-, *iso*-, and *anteiso*-chains) (linked to the carbon 3 of the FA): FA-β-O-L-Asp-L-Leu-L-Leu-L-Val-L-Val-L-Glu-L-Leu.

1.5.2 Bamylocin A

Bamylocin A from *B. amyloliquefaciens* LP03, an FA-O-heptapeptide with antifungal activity [4]. This secondary metabolite is composed of an FA linked by an ester bond to a heptapeptide.

FA-β-O-L-Glu-L-Leu-x-Met-x-Leu-x-Pro-D-Leu-L-Leu.

Cyclisation occurs as for surfactin, that is, by linkage to the carbon 2 of the FA.

1.5.3 Circulocins

Circulocins α-δ from *B. circulans J2154* are gFA-penta/hexa-peptides [5, 6]. These lipopeptides result from an ester linkage of a gFA and a pentapeptide or heptapeptide.

- Circulocin α: gFA-β-O-x-Thr-x-Phe-x-Ile-x-Dba-x-Asp
- Circulocin δ: gFA-b-O-x-Thr-x-Leu-x-Ile-x-Thr-x-Asn- x-Ala

1.5.4 Kurstakins

Kurstakins from *B. cereus* and *B. thuringiensis* are FA-N-heptapeptides with antifungal activity [2, 62–64]. The kurstakin family consists of partially cyclic heptapeptides associated with an amide bond with four different FAs (*iso*C11, *n*C12, *iso*C12, and *iso*C13). Lactonisation occurs between the carboxyl group of the terminal Gln and the hydroxyl group on the side chain of the Ser residue. The common peptide sequence is FA-b-N-Thr-Gly-Ala-Ser-His-Gln-Gln (Fig. 1). The NRPSs implied in kurstakin synthesis are encoded by the *krs* locus, which consists of six genes: (1) *krsE*, whose product is involved in the efflux of kurstakin; (2) the synthetase genes *krsA*, *krsB*, and *krsC,* which contain one, two, and four modules, respectively, with an E domain present in both the first and sixth modules; (3) *sfp*, which codes for a phosphopantetheinyl transferase; and (4) *krsD*, which mediates a type II thioesterase (Fig. 2).

1.5.5 Licheniformin

Licheniformin from *B. licheniformis* MS3 is an FA-N-heptapeptide containing an unusually long FA. This lipopeptide consists of a $C_{43}H_{87}$ branched fatty acid with a 13-fold repeated C_3H_6 unit linked by an amide bond to a heptapeptide: FA-β-N-Ala-Gly-Val-Asp-Ser-Gly-Tyr. Cyclisation occurs via an ester bond between the terminal Tyr and Asp [7].

1.5.6 Locillomycins

Locillomycins from *B. subtilis* 916 have both antibacterial and antiviral activities. These lipopeptides are FA-N-nonapeptides linked by an amide bond to a linear C13 or C15 FA, then cyclised by the formation of a macrolactone between the threonine hydroxyl and the valine carboxylate: FA-β-N-L-Thr-D-Gln-L-Asp-L-Gly-L-Asn-L-Asp-L-Gly-L-Tyr-L-Val (Fig. 1).

The nonlinear hexamodular NRPSs governing locillomycin synthesis are encoded by the *loc* cluster, which contains four genes: (1) *locD,* which encodes a polyketide synthase module; and (2) the NRPS *locA*, *loc*B, and *locC* genes, which contain one, three, and two modules, respectively, with an E domain being present in the second module. The latter function iteratively (Fig. 2) [8, 65].

To date, no studies on the NRPSs involved in the synthesis of the four lipopeptides, antiadhesin, bamylocin A, circulocins, and licheniformin, have been reported.

For a more detailed listing of nonribosomal lipopeptides from *Bacillus* sp., see the reviews of Aleti et al. [66] and Zhao and Kuipers [67].

2 Increasing Biodiversity by Genetic Engineering

As explained previously, NRPSs are composed of different modules, with different domains responsible for each step of amino acid integration in the peptide chain. Module or domain insertion, deletion, duplication, or exchange leads to multiple possibilities for biosynthesis in a design-based fashion of lipopeptides. Computational tools can be used to aid the combinatorial design of NRPS. Part mining for the collection and assembly of components can be linked to comparative analyses of gene clusters. However, the domain specificities and protein–protein interactions are complex and need to be studied to understand the molecular interactions between domains and modules.

2.1 Precursor Directed Biosynthesis

The first strategy, called precursor-directed biosynthesis, is not linked to the modification of the NRPS system, but the non-specificity of the A domain. Indeed, it has been observed that in natural biosynthesis, various products are formed in different proportions, some more abundantly than others. This is due to the substrate flexibility of the A domain, which can recognise similar amino acids. Thus, the feeding of monomers can enhance their concentration in the intracellular pool and change the final product of the NRPS, thereby enhancing their diversity. This strategy was frequently used, for example, with surfactin to increase the proportion of surfactin with valine at position 7 [68], to have an isoleucine residue at position 2 and/or

4 [69], to have an alanine residue at position 4 [70] or with iturin and fengycin to add a fluorinated non-proteinogenic amino acid [71, 72].

In the cases described above, the precursors were directly added to the culture medium and used for lipopeptide production. However, the feeding of some amino acids, not specifically precursors, also influences the final product by modifying metabolic pathways. Thus, independent of the recognition ability of the NRPS, but regarding the biosynthesis of branched FAs, the feeding of branched amino acids influences the proportion of even/odd FAs produced [73]. For surfactin, even *iso* FA is obtained with valine addition and uneven *iso* or *anteiso* FA is obtained with leucine or isoleucine addition.

Genetic engineering of the metabolic pathways involved in the biosynthesis of these precursors has also been investigated. Genetic engineering of the leucine pathway showed that the deletion of a global transcriptional regulator, *codY*, led to a 1.4-time increase in the surfactin form with valine at position 7 [74]. Similarly, the repression of different genes in the *bkd* operon led to an increase in the proportion of nC14 surfactin [74, 75].

However, there is still competition between the added and natural amino acids for cellular uptake and for A domain. To avoid this, a mutasynthesis can be performed [76–78]. In this case, the gene responsible for the synthesis of the natural precursor is deleted in the microorganism, and only monomer source is fed to control NRPS product synthesis. This technique was successfully used for some NRPS peptides, such as calcium-dependent antibiotics [78], and could be used for lipopeptides from *Bacillus* sp.

Other strategies to enhance lipopeptide diversity are based on modifications of the NRPS itself.

2.2 *Specificity Code Mutations*

The smallest modification that can be made to change the amino acid specificity is in the region coding for the specificity of the A domain itself. It was shown that eight amino acid residues in the active site of this domain allow for substrate specificity prediction. These residues, also known as ‘Stachelhaus code’ or ‘NRPS code’, are the basis for bioinformatic predictions [79, 80]. Thus, by modifying the residues of this Stachelhaus code, the substrate specificity can be changed. For surfactin, with site-directed mutagenesis, the A domain specificity changed from glutamic acid to glutamate and from aspartic acid to asparagine at positions 1 and 5, respectively [81].

2.3 *Domain Exchange*

Since an NRPS is composed of modules for each amino acid insertion, it is composed of domains for each step of the monomer aggregation, and the domain

exchange was one of the first techniques studied. Since the A domain is responsible for monomer insertion, its modification can lead to modification of the final product. This type of exchange was performed for the single A domain, but also for this domain combined with the others around it (condensation-adenylation or adenylation-thiolation) or for the whole module.

For surfactin, module swaps were performed, leading to variations in all seven amino acids, such as changes in the final amino acid from leucine to phenylalanine, ornithine, cysteine, and valine, or the replacement of an internal amino acid, such as leucine to ornithine [81–84]. In addition to domain exchanges, domain deletion leading to a shorter peptide chain in the lipopeptide also exists. Surfactin without Leu_2, Leu_3, Asp_5, or Leu_6 was produced by Mootz et al. [85] and Jiang et al. [86].

However, even if they could be increased, the yields obtained were lower than of the original products. Indeed, if the new domain is from the same biosynthetic gene cluster or from one that encodes a molecule of the same family, the exchange is more successful. Furthermore, a study performed by Bozhüyük et al. [87] showed that there is high flexibility between the C and A domains, and they created a new combination of domains called exchange units composed of A-T-C instead of C-A-T. Even if the NRPS of xenotetrapeptides was successfully redesigned, the yield obtained was still low [87]. Thus, these authors continued their studies and focused on C domains. Because the C domain catalyses the link between the amino acid of the preceding module and the current module, the concept of condensation subdomains was created. It separates the C domain into C_D and C_A, each of which is specific for the previous and current amino acids, respectively [15]. Thus, they redefined the exchange unit containing the C_A subunit, the A and T domains, and the C_D subunit. This redefinition of module boundaries allowed for the successful production of molecules through domain exchange with no loss in yield for the first time [88].

2.4 Starter Units and Tailoring Modifications

In addition to the exchange of domains within the NRPS, the initiation modules have been exchanged in the NRPS [89, 90]. Even if the downstream compatibility remains and must be addressed, upstream restraints do not exist in the initiation module. The addition of tailoring domains can also be achieved. They can be added to the domains as subdomains, thus allowing site-specific modifications. Selective methylation [91], halogenation, glycosylation, acylation, and sulphation [78] were performed on NRPS peptides and could be applied to lipopeptides from *Bacillus* sp.

3 Bioproduction in a Controlled Environment

3.1 Genetic Engineering Overproduction

The production process and its improvements are discussed later. First, overproduction through genetic engineering is covered. Because it is at the source of production, strain modification is important and influences the optimisation of the production process.

3.1.1 Transcription

Promoter exchange is one of the most common methods used to overproduce lipopeptides. It consists of the exchange of the original gene promoter by an induced-specific, constitutive, or stronger promoter. This method has been used for surfactin, and it has been shown that the use of an inducible promoter leads to a 17-fold increase in surfactin production [92]. However, the use of constitutive promoters depends on the original promoter. Indeed, if the bacterial strain is a strong surfactin producer, then the production will decrease with the change, whereas if the bacterial strain is a weak surfactin producer, the production will increase with the change [93]. Similarly, for iturin and fengycin, changes in the promoter also lead to an increase in production [94–97].

Additionally, transcriptional regulatory genes also influence lipopeptide production by regulating the NRPS operon of the lipopeptide or other mechanisms that indirectly impact their production. Positive regulators, such as PerP [98], DegQ [94, 99], ComA, and SigA [100], or negative regulators, such as CodY [101], Rap [102], SinI [103, 104], Spx [105], and AbrB [97], have been found to be involved in lipopeptide production by *Bacillus* sp. It has been shown that the cell density-dependent quorum-sensing system influences surfactin production through the ComQXP quorum-sensing locus [106] as well as ComX and PhrC [107].

3.1.2 Increase in Precursor

Coutte et al. [101] showed that the feeding of leucine to the culture medium led to a three-fold increase in surfactin production. Comparable results were obtained for the iturin family with the addition of its amino acid to the culture medium [108–111]. It was thus shown that the addition of lipopeptide precursors to the culture medium for their synthesis led to increased production.

Furthermore, as mentioned before, to avoid the feeding of monomers that enhance their concentration in the intracellular pool, metabolic engineering can be used to modify the metabolic pathways involved in their production. This approach was used by Dhali et al. [74] and Wang et al. [75]. From the modelling of the metabolic pathway of leucine, gene knockout was selected and led to a 5.8-time

increase in surfactin production [74]. Similarly, the knockout of three negative regulators of glutamate metabolism leads to an increase in surfactin production [75].

This precursor increase strategy was also used for the fatty acid part of the molecule. The synthesis of branched-chain α-ketoacyl-CoA and malonyl-ACP increased [74, 112]. Wu et al. [112] also overexpressed the entire FA synthase complex.

3.1.3 Excretion

From a production point of view, even if the NRPS assembly of a lipopeptide is high, if its excretion does not follow, the final product recovered will not be satisfactory.

Despite their nature, the efflux of lipopeptides is mediated by protein transporters. Li et al. [113] showed that an increase in surfactin export was observed after the overexpression of three lipopeptide transporters.

3.1.4 Degradation

As for excretion, a degradation that could occur after production would diminish the final lipopeptide production capacity. This problem was confirmed by the observed decrease in surfactin concentration during fermentation [114, 115]. This could result from the action of a protease produced by the bacterial strain that produces the lipopeptide [115]. This degradation process could be developed by the *Bacillus* strain for two reasons. The first is the use of the product as a carbon source after the depletion of other sources. The second is the degradation of the lipopeptide because of its possible inhibitory effect at higher concentrations [114].

3.2 Bioprocess Optimisation

As lipopeptides are surfactants, they decrease the energy needed for foam formation by increasing the stability of the bubbles in the gas–liquid dispersion. Indeed, by reducing the surface tension, the coalescence of bubbles in the foam is inhibited. The most common procedure to avoid foam is to add anti-foam to the culture medium. However, the addition of anti-foam to the culture medium has many drawbacks. It is costly and can have a negative effect on cell growth. Furthermore, since it is added to the culture medium, it adds a purification step because it must be removed. Thus, alternatives are needed to address foam production during lipopeptide production.

3.2.1 Foaming Processes

Because the lipopeptides are placed at the gas–liquid interface, this foam formation can be seen as a drawback because of the loss of surfactant production. However, it can also be used as an advantage because the foam can be recovered and used as the first purification step. Furthermore, the continuous extraction of lipopeptides from the culture avoids any possible feedback inhibition from the products. A lipopeptide production with a foam fractionation strategy was put into place, with the fermenter linked to foam columns. The foam filling the headspace of the fermenter was collected into a sterile vessel. However, the outgoing flow rate needs to be tightly controlled because it can block the exit filters or even overflow out of the fermenter. Furthermore, a drawback of these foaming processes is that the culture medium and cells are trapped in the foam, leading to a decrease in production. Despite these inconveniences, foam fractionation has been used for lipopeptide production, mostly for surfactin. Cooper et al. [116] used a batch process composed of a fermenter with a collection vessel in the air exhaust line for surfactin production. The foam was continuously carried away with the air. The foam column can also be inserted into the fermentation vessel head plate [117]. The complexity of the outgoing foam flow rate control was highlighted in Davis et al.'s experiment [117]. Higher aeration of the culture through mixing and air supply led to better surfactin production and foam production that carried away the culture medium. This problem was addressed by Yeh et al. [118] with a cell recycler and surfactin precipitator to maintain the culture medium in the fermenter. The use of two foam collectors has been investigated to enhance foam recovery [119]. Guez et al. [120] adapted this method for mycosubtilin production in a fed-batch process. Even if less foam is produced by this lipopeptide because of its lower surfactant abilities compared to surfactin, foam fractionation is still needed. A feeding rate law was computed for fed-batch processes [121], limiting the feed rate to 0.1 h^{-1} to avoid culture medium loss in the foam. A continuous process linked to foam fractionation [122] can solve the problems mentioned above and lead to a culture that can last up to 100 h of fermentation.

3.2.2 Non-foaming Processes

Solid-State Fermentation

Historically, *B. subtilis* has been used in alkaline food fermentation throughout the world and produces lipopeptides in addition to fermentation [123, 124]. Since it is a possible production method, and most of the cheap substrates, such as waste or by-products from the agro-industrial field, are used in solid-state fermentation, solid-state is a surging method used for lipopeptide production. It has been used for a long time with soybean curd residue [125, 126], and a lot of research has been performed for surfactin production with various wastes, such as soybean flour, rice straw, corn

steep liquor, olive oil mill waste, brewery wastewater, beet molasses, apple peel extract, carrot peel extract, and glycerol [127–131].

Immobilised Cells

Cells immobilised on solid particle carriers in a liquid fermenter have been used for lipopeptide production. Reduced foam formation, in addition to enhanced lipopeptide production, was observed. Activated carbon, agar, and expanded clay have been added to the culture medium as solid carriers for the bacteria that produce surfactin [132]. Polypropylene particles of low density have also been used to produce surfactin and fengycin [133, 134]. These low-density particles were used in a three-phase inverse fluidised bed bioreactor. This resulted in a high oxygen transfer rate, but foam production was also observed. It has been reported that oxygenation can influence the production of different lipopeptide families by *B. subtilis* [135]. Moderate oxygenation leads to the production of a mix of families, whereas high oxygenation leads to the production of surfactin alone.

Rotating Disc Reactor

Bioreactors containing rotating discs partially immersed in the culture medium are called rotating disc bioreactors. The aeration is made through the rotation of the discs and the contact between the air and the liquid film on their surface, allowing no foam production. Bacterial cells are mainly attached to the discs. This technique is often used in wastewater treatment since it is easy to use and to scale up [136], but, until now, not much for the production of molecules of interest. Chtioui et al. [137] produced two lipopeptides, surfactin and fengycin, in such a bioreactor. As it has a low oxygen transfer rate, they later modified the rotating disc bioreactor by adding mechanical agitators to enhance the aeration of the culture medium, thus improving fengycin production [138].

Biofilm Reactors

In biofilm bioreactors, structured packing, which allows cell adhesion and biofilm formation, is added to the same vessel above the culture medium or in a separate vessel. In this type of reactor, the trickling of the culture medium on the packing enhances oxygenation and thus promotes lipopeptide production. In addition, it can reduce foam formation during lipopeptide production. Indeed, the cells, at first in suspension in the culture medium, form a biofilm on the packing due to the continuously recirculated culture medium on it. Furthermore, the oxygenation of the culture medium takes place in a liquid-to-gas dispersion type instead of a gas-to-liquid dispersion, leading to less foam formation. Biofilm formation is strongly dependent on the liquid distribution style and flow rate. For the liquid distribution, a distributor plate instead of a single injection point leads to a slower liquid velocity,

and thus, an optimal biofilm dispersion on the packing and better aeration with less foam formation [139, 140]. Another method to optimise biofilm formation is to genetically modify the strains to obtain filamentous growth or exopolysaccharide production [141]. To optimise this process, biofilm dynamics analysis can be performed directly through X-ray tomography analysis of the packing [139], or indirectly through the cells released from the biofilm in the culture medium using flow cytometry or other single-cell techniques [142, 143]. This type of biofilm reactor has been used by Zune et al. [140] and Brück et al. [144] for lipopeptide production, and, more specifically, surfactin.

Air Liquid Membrane Contactors

Air liquid membrane contactors are composed of an organic high-area membrane, separating the compartment flushed with air from the one flushed with the culture medium. The oxygen transfer is made through this membrane from the air compartment to the liquid compartment, avoiding foam formation. This bioreactor has been used for surfactin production [145]. Optimisation of this technique was conducted, with a change in the culture medium composition, from a bacterial growth optimised medium to a surfactin production optimised medium [146]. Another advantage of the membrane is the possibility of coupling it with the first step of purification, such as cell filtration. Thus, a continuous process can be put in place with increased productivity [147]. However, fouling of the membrane with cells or produced molecules is a disadvantage, as is the price of the membrane.

3.3 Purification

The downstream processes are responsible for more than half of the production cost of a molecule. Thus, the extraction, recovery, and purification techniques for lipopeptides have been thoroughly studied. These studies were mainly based on *B. subtilis* cultures, with surfactin, fengycin, and iturin production.

3.3.1 Acid Precipitation

Purification using acid precipitation is the oldest lipopeptide purification technique used. After culture, the culture medium is centrifuged to remove the cells. Then, the lipopeptides are precipitated by acidification at pH 2 overnight at 4°C, followed by the addition of chloric or sulphuric acid. Centrifugation allows the separation of the precipitated molecules. Finally, the extraction is performed using organic solvents (methanol, chloroform, acetonitrile, and acetone) [148]. The solvent can be used alone, but better results were obtained through their coupling, mostly with methanol/chloroform (2:1 v/v) [149]. Because it is acid precipitation, this technique is only

suitable for negatively charged lipopeptides, such as surfactin. Furthermore, it is not possible to continuously couple this technique with production. Because the purity is frequently insufficient, multiple steps of solvent extraction should be used to enhance this procedure, or it should be coupled with another technique.

3.3.2 Foam Fractionation

As mentioned previously, a foam fractionation production technique allows the recovery of the foam containing lipopeptides during fermentation [117, 120, 121]. Therefore, this process combines production and purification. For a continuous approach, the foaming rate, linked to agitation, must be closely monitored. However, even if a semi-batch mode allows up to 100% recovery, the continuous mode allows only 70% recovery [150].

3.3.3 Adsorption

Another technique that allows continuous purification during the production process is the adsorption of lipopeptides on various supports [151, 152]. An adsorption column is coupled to the fermenter, and its physicochemical properties cause only the lipopeptide to adsorb. The absorption column can be composed of a microporous PVDF hollow fibre membrane or active carbon. The purity obtained is always acceptable (higher than 75%), but the recovery rate depends on different factors, such as the initial concentration in the culture medium or the temperature.

Chromatographic methods, even if not applicable for high volumes, lead to the best purity and the separation of different lipopeptide forms if several families are produced at the same time in the fermenter. Chromatographic derivative methods were developed for larger volumes, with the same family separation and a lower, but still above 70%, purity [153].

3.3.4 Membrane Ultrafiltration

Surfactants can form micelles above their critical micelle concentrations. They are aggregates of the surfactant, and thus have a larger diameter than the individual free molecules. Because lipopeptides are subjected to this phenomenon, this size enhancement can be used to purify them. A membrane with a molecular weight cut-off of 10 to 100 kDa can retain surfactin and mycosubtilin, thus enhancing their concentration in the retentate [154]. Afterwards, resolubilisation in an organic solvent breaks down the micelles into individual molecules. The pH of the solution, membrane type, and lipopeptide concentration affect the process. Membrane fouling with micelles can decrease the permeate flux [155]. Since surfactin micelles can interact with bivalent positive ions, such as $CaCl_2$, their size can be enhanced and a larger molecular weight cut-off can be used for the membranes [156].

3.3.5 Liquid/Liquid Solvent Extraction

Liquid membrane pertraction was used by Dimitrov et al. [157] and Chtioui et al. [133] with n-heptane/1-octanol (50/50) and n-heptane as solvents, respectively. This process extracts and purifies lipopeptides, with recovery from 75% to 97% depending on the solvent and pH. A solvent extraction assisted with ultrasound treatment enabled the extraction of iturin and surfactin, allowing up to 100% recovery [158].

3.3.6 Hybrid Methods

Most of the methods described above are not used alone, but in combination with one another to provide higher recovery and purification rates. Membrane ultrafiltration is mostly used for precipitation or adsorption. Membrane ultrafiltration can be carried out in one or two stages and can be combined with diafiltration or precipitation to give a purity of 70% to 98% [154, 155, 159, 160]. It can also be combined with a preceding salting-out using ammonium sulphate and ethanol [161], or adsorption on various resins, such as XAD-7 [162].

4 Biodiversity and Physicochemical Properties

The amphiphilic structure of the lipopeptides produced by *Bacillus* sp. confers interesting physicochemical properties, including surface-tension reduction and self-assembly capacity. Some are also able to bind to ions. The distribution of the hydrophobic and hydrophilic moieties, the presence of charge(s), as well as the cyclic or linear form of the peptide part, the ring closure type, and the length and configuration of the lipidic part highly affect these properties.

Owing to these physicochemical properties, these lipopeptides show promising applications, mainly in the environmental sector as oil recovery enhancers, xenobiotic hydrocarbon biodegradation activators or as metal recovers from soil or water, and in pharmaceutical areas for formulating stable microemulsions for drug delivery (for a recent review on lipopeptide applications, see [163]) (Fig. 3).

In literature, the surfactin family is the most extensively studied lipopeptides in terms of physicochemical properties (see our recent review [164]), followed by the iturin family. The other families are much less investigated, probably due to the low production yield limiting the amount of high-purity compounds necessary for physicochemical experiments. This is particularly true for locillomycin [1, 8].

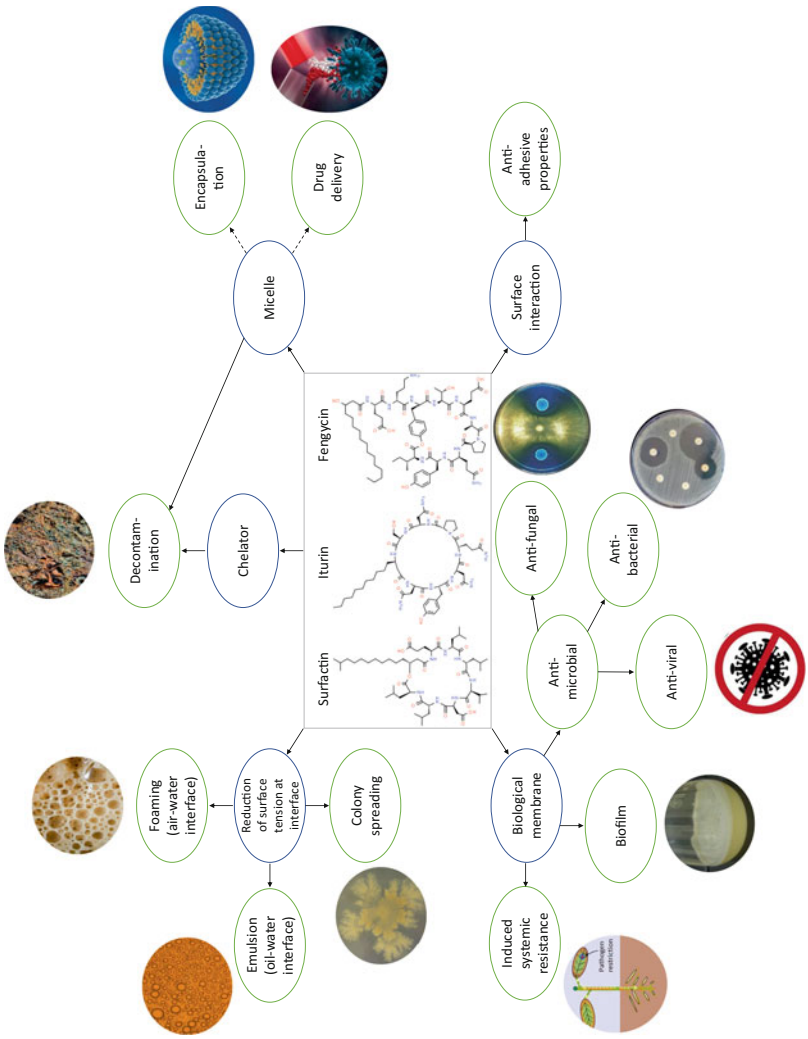


Fig. 3 Schematic representation of the link between the physicochemical properties (in blue) of the lipopeptides produced by *Bacillus* sp. and their biological properties (in green). Dotted lines represent a potential biological application

Table 1 Critical micellar concentration (CMC) and surface tension at the critical micellar concentration (γ_{CMC}) of the main lipopeptides produced by *Bacillus* sp. at an air–water interface

Lipopeptides	CMC (μM)	γ_{CMC} (mN/m)	Reference
Surfactin (mixture of homologues)	9–220	30–31	[20, 165–167]
Linear surfactin C14	374	36–37	[168]
Surfactin C13	83.6	36.4	[169, 170]
Surfactin C14	65.1	33.5	[169, 170]
Surfactin C15	19.5	31.9	[169, 170]
Lichenysin	22	29	[20, 165]
Pumilacidin C14	94	56	[171]
Iturin A (mixture of homologues)	43	54.5	[167]
Iturin A C14	141	45.4	[172]
Iturin A C15	88	45.6	[172]
Iturin A C16	79	41.9	[172]
Iturin A C17	68	38.3	[172]
Mycosubtilin	37	55	[167]
Bacillomycin D	170	53	[167]
Bacillomycin F	27	50.5	[167]
Iturin C	80	49.6	[167]
Bacillomycin L	160	46	[167]
Fengycin (mixture of A, B, and homologues)	4.6	43	[173]
Kurstakin	162	33	[174]

4.1 Surface Activity

The surface-active property refers to the capacity of a surfactant molecule to reduce the surface or interfacial tension between a gas and a liquid or between two immiscible liquids, respectively. This characteristic correlates with the foaming or emulsifying properties of the surfactant.

Lipopeptides from the surfactin family are the most active in reducing the surface tension (γ) of the air–water interface (Table 1). A reduction from γ~72 mN/m to values between 29 and 56 mN/m depending on the structure of the surfactin and the experimental conditions were reported. The native surfactin [166], and lichenysin with a Gln instead of Glu at position 1 in the peptide cycle are the most active [165] and pumilacidin in the presence of Leu_4 instead of Val_4 is less active [171]. The length and branching configuration of the alkyl chain influence surface tension. A longer chain is more efficient [170] and the normal configuration is more active than the *iso* one, which is more powerful than the *anteiso* [175].

Iturinic lipopeptides globally show a lower surface tension reduction power than the surfactin family, with γ values between 46 and 55 mN/m at an air–water interface (Table 1) [167]. Compared to iturin A, mycosubtilin with an inversion of Ser and Asn between positions 7 and 6, and bacillomycin D (with the sequence L-Pro/L-Glu/D-Ser/L-Thr for positions 4 to 7) have similar behaviour, bacillomycin F with a Thr residue instead of Ser at position 7 had intermediate activity, whereas iturin C with

Asp instead of Asn at position 1 and bacillomycin L (with L-Asp at position 1 and L-Ser/L-Gln/D-Ser/L-Thr at positions 4 to 7) had the highest activity. For iturin A, an increase in chain length from C14 to C17 globally decreases the γ_{CMC} value [172], in accordance with the results of Habe et al. [176].

It is worth noting that surfactin and iturin can form a complex at a surfactin-iturin molar ratio of 2:3 that exerts a synergistic effect on the dynamic surface tension reduction [177].

The surface activity of the fengycin family has not been thoroughly investigated. A mixture of fengycin A and B homologues has an air–water surface tension reduction capacity [173] between those of surfactin and iturin families (Table 1).

The same trend between the three main families of lipopeptides produced by *B. subtilis* was also observed at the dodecane/water interface. Surfactin is more effective than fengycin, which is better than iturin A in reducing interfacial tension [178].

By reducing the surface tension of water to 33 mN/m [174], kurstakin has surface-active performance comparable to that of surfactin.

No data are available for locillomycin and other lipopeptides produced by *Bacillus* sp.

Besides their activity at an air–liquid or liquid–liquid interface, some lipopeptides are able to adsorb on solid surfaces and modify their surface hydrophobicity. The effect depends on the nature of the lipopeptide, its concentration, and the solid support. On polystyrene and stainless steel, kurstakin (produced by *Enterobacter cloacae* C3 strain) and surfactin decreased the surface hydrophobicity, resulting in a decrease in hydrophobic interactions with the cell wall of microorganisms and consequently their adhesion to these solid supports [179, 180]. The adsorption of fengycin onto stainless steel increases its hydrophobicity up to its CMC and decreases it at higher concentrations [173]. Surfactin, mycosubtilin, and iturin A decrease the hydrophobicity of Teflon [173]. Surfactin and mycosubtilin are also able to increase the wettability of agar solid surfaces, which facilitates bacterial swarming [181].

Moreover, some lipopeptides have also been shown to modify the surface hydrophobicity of their producing bacteria, and consequently, to impact bacterial adhesion to solid surfaces. The effect of surfactin and iturin A on the surface hydrophobicity of *B. subtilis* was observed. Their effect depends on the initial bacterial hydrophobicity, as well as the lipopeptide type and concentration [182]. Surfactin and iturin A enhance or decrease the bacterial surface hydrophobicity, making it less or more hydrophobic. Surfactin is more efficient than iturin A. Kurstakin has also been shown to increase the surface hydrophobicity of *Acinetobacter haemolyticus* strain 2SA, facilitating the contact of hydrophobic substrates with this bacterium [174].

4.2 Self-assembly

One common feature of surfactants is their ability to self-assemble in aqueous media at a defined concentration threshold called the critical aggregation concentration (CAC). In most cases, the self-assembly is a sphere-like micelle (in this case, CAC can be named CMC for critical micellar concentration) but other self-assembled nanostructures like wormlike micelles, unilamellar bilayers, and larger aggregates can co-exist depending on the surfactant concentration, pH, temperature, ionic strength, and the nature of the ions (see the review of Carolin et al. for further information [163]). A lower CAC value correlates with higher efficiency for solubilising hydrophobic compounds.

Among the three main cyclic lipopeptide families produced by *B. subtilis*, fengycin has the lowest CMC value, followed by surfactin, and iturin A (Table 1) [167, 173]. The synergistic effect between surfactin and iturin A observed for surface tension reduction was not observed for the self-assembly property [177]. Kurstakin has a lower propensity for self-association (Table 1) [174]. No data were found for the other lipopeptides produced by *Bacillus* sp.

Within the surfactin family, a small structural difference can significantly affect CMC. The presence of a methyl ester on the Glu residue or the replacement of the Glu residue with Gln, as in lichenysin, decreases the CMC (Table 1) [20, 165]. The linearisation of the peptide cycle [168] and the presence of a Leu_4 instead of Val_4, as in pumilacidin [171] is unfavourable to the propensity for self-assembly (Table 1). As commonly observed for surfactants, a longer FA favours aggregational behaviour (Table 1) [169, 170].

Among iturin members, the CMC value is also greatly influenced by its structure in the following order: bacillomycin F < mycosubtilin < iturin A < iturin C < bacillomycin L < bacillomycin D (Table 1) [167]. The presence of a carboxyl group on an aspartyl (iturin C and bacillomycin L) or glutamyl (bacillomycin D) residues seems to be unfavourable for self-aggregation. The FA length can also have an influence since mycosubtilin and bacillomycin F have a C16 or C17 chain length, whereas iturin A, C, and bacillomycin D and L have a C14 or C15 chain. In the case of iturin A, a longer chain length also favours a decrease in the CMC value (Table 1) [172].

The nanostructure of the self-assemblies of some lipopeptides has been investigated and shows contrasting results. Surfactin and plipastatin self-assemble into spherical micelles, whereas mycosubtilin forms extended nanotapes composed of a stacking of lipopeptide bilayers [183]. The branching of the lipid chain of mycosubtilin can be the origin of this peculiar self-assembly behaviour. It was also shown that at a concentration well above its CMC, iturin A forms vesicles with a lamellar organisation [184].

4.3 Ion Complexation

Some lipopeptides can bind ions, which can be beneficial for the decontamination of polluted soil or water.

The presence of electrostatic charges in the peptide cycle, such as the two carboxylic groups in surfactin, is favourable for this interaction. Surfactin is indeed able to bind divalent cations, and this property is dependent on the pH; a higher pH value increases its binding ability. The binding is not only driven by the presence of the charges, but also by the steric hindrance of the cation and the size of the claw formed by the two acidic side chains [20]. This explains the better selectivity of surfactin for Ca^{2+} than for Mg^{2+}. Surfactin is also able to bind monovalent cations, such as Rb^{+}, but with a lower affinity than divalent cations [185]. The ability of surfactin to extract Ca^{2+} and Rb^{+} from an aqueous phase to give lipid-soluble complexes has suggested that surfactin can be a mobile carrier of cations [185], which could have an impact on its biological properties.

Lichenysin can also complex divalent cations and has been demonstrated to be a better divalent cation chelating agent than surfactin [165]. This effect is attributed to the increased accessibility of the carboxyl group to the cation in the case of lichenysin [186]. The complexation of divalent cations with the lipopeptide in a molar ratio of 2:1 for lichenysin leads to the formation of an intermolecular salt bridge that is stronger than the intramolecular complexation in a 1:1 ratio with surfactin [165, 186].

Iturin A can also bind alkali metal ions with the following ion selectivity order: Na+ > K+ > Rb+ [187]. It is hypothesised that alkali metal ions bind to either one of the two β-turns in the peptide ring, with the carbonyl oxygen moieties acting as chelating atoms.

5 Biodiversity and Biological Activities

Bacillus lipopeptides are also able to strongly interact with biological membranes [188]. This property is the basis of most of their biological activities, including antimicrobial and antiviral activities, cytotoxicity, and the ability to induce systemic resistance in plants (Fig. 3). In addition, some other specific targets are involved in biological activities, such as anticancer, anti-inflammatory, and immunomodulatory activities.

5.1 Antimicrobial and Antifungal Activity

The best described activity of *Bacillus* lipopeptides is their antimicrobial properties. Indeed, iturin and fengycin are well-known antifungal compounds. The mode of

action generally includes membrane destabilisation and pore formation, leading to cytosol leakage and subsequent cell death [189–194]. However, the detailed interactions between lipopeptides and cell membranes remain to be elucidated. Interestingly, it seems that the lipid composition of the targeted membrane affects the potency of the lipopeptide [195–197]. Iturin and mycosubtilin inhibit various fungal phytopathogens, such as *Fusarium graminearum, Monilinia fructicola, Colletotrichum gloeosporioides, Aspergillus flavus*, and the oomycete *Phytophthora infestans* [198–202]. Furthermore, fengycin is effective against *F. graminearum* and *M. fructicola* in addition *to Rhizopus stolonifer, Magnaporthe grisea*, and *Rhizoctonia solani* [196, 199, 200, 202–204]. Fengycin also inhibits the synthesis of mycotoxins by *F. graminearum* [200]. In addition to their antifungal activities, some articles have reported the antibacterial properties of these compounds against the phytopathogens *Xanthomonas campestris* pv. *cucurbitae, X. axonopodis* pv. *vesicatoria, Pectobacterium carotovorum*, and *Ralstonia solanacearum* [194, 205, 206]. Finally, the synergistic effect of these lipopeptides with surfactin has been reported. The combination of the latter with either iturin variants (bacillomycin D or mycosubtilin) or fengycin improves the biocontrol of *Botrytis cinerea, F. oxysporum,* and *P. infestans* [207–209]. Even though surfactin has been described as an antimicrobial agent *sensu stricto*, the concentrations required (from 50 to 200 μM) are far higher than those found under natural conditions [210–213]. Nevertheless, surfactin can alter other microorganisms by interfering with the establishment process, as reported for *Pseudomonas syringae* and *R. solanacearum* [214–217]. Moreover, the roles of surfactin, independent of its surfactant properties or membrane destabilisation ability, have been reported. Indeed, surfactin inhibits the erection of aerial hyphae and subsequent spore formation by *Streptomyces coelicolor* by altering the expression of developmental genes required for secondary mycelium development [218, 219]. *Moreover, surfactin has been reported to block the synthesis of* β-glucan and weaken the fungal cell wall [220]. *These more subtle activities support the multifaceted and complex biological activities of Bacillus cyclic lipopeptides (CLPs).*

So far, the antimicrobial activities of these lipopeptides have been mainly carried against phytopathogens, as producing strains are potent biocontrol agents. Nonetheless, antimicrobial properties of human pathogens have been reported. Mycosubtilin shows high anticandidacidal activity [221]. Surfactin is effective against *Legionella pneumophila*, and *Listeria monocytogenes* and lichenysin showed a synergetic effect when combined with the gemini surfactant $C_3(LA)_2$ towards *Escherichia coli, P. aeruginosa, Klebsiella pneumoniae, Yersinia enterocolitica, L. monocytogenes,* and *Candida albicans* [222–225].

5.2 Cytotoxicity

As lipopeptides produced by *Bacillus* sp. affect the cell membrane, these lipopeptides can be toxic to cells at high concentrations and can cause cell death

through membrane disruption. The interaction of surfactin with biological membranes can be linked to the length of the FA, with the highest cytotoxicity observed for longer chains than for smaller chains [226].

Surfactin could be considered safe as its acute toxicity in mice was determined with an LD_{50} higher than 100 mg/kg by intravenous administration [227]. When administered orally to mice, up to 10 mg/kg of surfactin in the long-term application did not show apparent toxicity [228]. A study has determined the no-observed-adverse-effect level of surfactin to be 500 mg/kg, following oral administration in rats [229] and the same study found no mutagenic toxicity of surfactin. Iturin was also found to be safe and non-toxic for oral consumption at concentrations lower than 5 g/kg and did not cause organ damage or abnormal blood indices in mice after feeding for 28 days at a dose of 2 g/kg [230]. Other studies have shown that iturin A had an IC_{50} of approximately 50 mM in BRL-3A rat liver cells [231] and that exposure to fengycin at 200 mg/L did not have any adverse effect on the viability of MRC-5 human normal lung fibroblasts [205].

The haemolytic activities of surfactin and surfactin-like lipopeptides were further studied for potential medical applications of surfactin. A study by Dehghan-Noudeh et al. [232] concluded that surfactin has haemolytic activity at concentrations above 0.05 g/L, which is lower than that of chemical surfactants but could limit the use of surfactin in medical applications. Symmank et al. produced a new lipohexapeptide with reduced toxicity against erythrocytes and enhanced antibacterial activity [233]. Linear analogues of surfactins were synthesised by Dufour et al., who showed no significant haemolysis activity compared with cyclic surfactin, demonstrating the importance of the cyclic structure to disrupt cellular membranes [168]. More recently, surfactin analogues were chemically synthesised, and one of the linear analogues with a shorter peptide chain had increased antiviral activity and lower haemolytic activity than surfactin. These studies indicate that new surfactin analogues could have the same or increased biological activity with a lower haemolytic activity, which could greatly increase the usage and impact of surfactin-like lipopeptides in medical applications. The haemolytic activity of iturin has also been reported, with iturin A and bacillomycin L exhibiting complete haemolysis at 20 mg/L and 10 mg/L, respectively, when iturin C did not show any effects on erythrocytes [234]. The haemolytic activity of iturin A was further studied by Aranda et al. [235], who found that iturin A released haemoglobin at approximately 10 μM, and that at 25 μM, haemolysis was complete. They demonstrated that iturin A causes haemolysis of human red blood cells via a colloid-osmotic mechanism. Finally, a study by Vanittanakom and Loeffler showed that fengycin has a 70-fold lower haemolytic activity than iturin A [30].

5.3 Antiviral Activity

Lipopeptides produced by *Bacillus* have been shown to have antiviral properties, with studies focusing on the antiviral activity of surfactin. Surfactin has been shown

to inactivate a wide range of enveloped DNA and RNA viruses, including Semliki Forest virus, herpes simplex virus-1 and -2, vesicular stomatitis virus, simian immunodeficiency virus, pseudorabies virus, Newcastle disease virus, transmissible gastroenteritis virus, porcine epidemic diarrhoea virus, and human coronavirus 229E, Middle East respiratory syndrome coronavirus, recombinant severe acute respiratory syndrome coronavirus, chikungunya virus, Nipah virus, Dugbe virus, Zika virus, Crimean-Congo haemorrhagic fever virus, influenza A H1N1 and H3N2, and Ebola virus [236–242]. Surfactin was found to be less effective or ineffective against non-enveloped viruses, such as feline calicivirus, murine encephalomyocarditis virus, porcine parvovirus, infectious bursal disease virus, and coxsackievirus B3 [236, 237, 239]. These studies suggest that at a high concentration, surfactin disintegrates the lipidic envelope of the virions. However, at a low concentration, surfactin could act as a membrane fusion inhibitor by insertion into the viral envelope and stabilisation of the positive curvature of the envelope [237, 239, 242]. Furthermore, the composition of the envelope, the number of carbon atoms in the FA of surfactin, and the charge of the peptide moiety influence the antiviral activity of surfactin [237–239, 241]. In vivo experiments have shown that oral administration of surfactin to piglets could protect them against porcine epidemic diarrhoea virus infection; however, prophylactic treatment failed to protect mice against severe acute respiratory syndrome coronavirus [237, 242]. Finally, a study by Yuan et al. evaluated surfactin analogues obtained by chemical synthesis and found an analogue with the same antiviral activity as surfactin but with lower haemolytic activity [241].

5.4 Anticancer Activity

Lipopeptides produced by *Bacillus* sp. have been shown to have cytotoxic effects on many different cancer types, including breast cancer, colon cancer, leukaemia, hepatocellular cancer, cervical cancer, and lung cancer. Lipopeptides from *Bacillus* sp. have been reported to induce apoptosis and arrest the cell cycle in different tumour cell lines. Different mechanisms have been suggested for their anticancer activity: inhibition of the nuclear factor-κB (NF-κB) activator protein 1; phosphatidylinositol 3-kinase/Akt and the extracellular signal-regulated kinase signalling pathway [243, 244]; cell arrest at G(2)/M phase [245], through reactive oxygen species-mediated mitochondrial/caspase pathway [246, 247]; and disturbance of the cellular FA composition of the cancerous cells [226]. Liu et al. [226] demonstrated that the length of the FA affected the cytotoxicity of surfactin on cancer cells, with the cytotoxicity increasing with the length of the FA.

Human breast cancer cells (MCF-7) were successfully inhibited by surfactin in a dose-dependent manner, with an IC_{50} of approximately 10 μg/mL at 24 h [248]. Dey et al. showed that iturin A also inhibited human breast cancer in vitro using the cell lines MDA-MB-231 and MCF-7 and in vivo using mouse xenograft models, and induced apoptosis by disrupting the Akt pathway [249]. A study by Zhao et al. also

showed that iturin A had an IC_{50} of approximately 60 μM at 48 h in MCF-7 cells [230].

Studies have also described the anticancer activity of surfactin, fengycin, and iturin in colon cancer. Kim et al. reported the inhibition of LoVo cell proliferation by inducing pro-apoptotic activity and stopping the cell cycle with an IC_{50} of 26 μM at 48 h [243]. Sivapathasekaran et al. isolated isoforms of surfactin and fengycin from a marine *B. circulans* DMS-2, which inhibited HCT-15 and HT-29 cell lines with an IC_{50} of 80 μg/mL against HCT-15 cells and 120 μg/mL against HT-29 cells [250]. These results were confirmed by Cheng et al., who showed that fengycin had an inhibitory effect on HT-29 cells at 20 μg/mL at 72 h and could induce HT-29 cell apoptosis and stop the cell cycle [251]. Additionally, iturin A had an IC_{50} of approximately 70 μM at 48 h in Caco-2 cells [230].

Lipopeptides from *Bacillus* sp. also have anticancer effects on leukaemia. Indeed, a surfactin-like lipopeptide purified from *B. subtilis* natto T-2 exhibited an inhibitory effect against human K562 leukaemia cells and caused dose-dependent apoptosis of K562 myelogenous leukaemia cells through cell phase arrest [252]. In another study, a mixture of iturin homologues was shown to inhibit K562 cells, with an IC_{50} of 65.76 μM [230].

Surfactin has also been shown to have anticancer activity against Ehrlich ascites carcinoma [253], hepatocellular carcinoma, cervical cancer, human oral epidermoid carcinoma, pancreatic, and rat melanoma cancer [226]. Fengycin has been found to inhibit the proliferation of the human lung cancer cell line 95D and the growth of xenografted 95D cells in nude mice [247]. Finally, a study by Zhao et al. [230] showed that iturin inhibited the growth of human liver cancer (HepG2 cell line) and lung cancer (A549 cell line).

5.5 *Anti-inflammatory Activity*

Studies on the anti-inflammatory activity of *Bacillus* sp. lipopeptides have mostly focused on surfactin and have demonstrated the potential of surfactin as a new anti-inflammatory molecule. Several studies have demonstrated that surfactin can inhibit the inflammatory effects of lipopolysaccharide (LPS) on eukaryotic cells. As recently reviewed by Zhao et al., the mechanisms involved in this anti-inflammatory activity are thought to be interaction with cytosolic phospholipase A2 (PLA2), modulation of the TLR4 and NF-κB cell signalling pathways, inhibition of lipoteichoic acid-induced NF-κB, activation of signal transducer and activator of transcription-1, and increased phosphorylation of signal transducer and activator of transcription -3 [254]. Kim et al. were the first to demonstrate the anti-inflammatory activity of surfactin by selective inhibition of cytosolic PLA2, which could suppress inflammatory responses [255]. Surfactin also inhibits the LPS-induced transcription of inflammatory mediators, such as IL-1β, inducible nitric oxide synthase (iNOS), and reducing nitric oxide (NO) production [256]. Surfactin treatment reduces plasma endotoxin, TNF-α, and NO levels in response to septic shock in rats [228]. A study

comparing the anti-inflammatory effects of C13, C14, C15, and C16 surfactin found that C14 surfactin was the most efficient at inhibiting NO production, iNOS, and monocyte chemoattractant protein 1 (MCP-1) [257]. The interaction of lipid A with LPS-binding protein (LBP) was shown to be suppressed by surfactin [258]. Other anti-inflammatory activities of surfactin include the downregulation of LPS-induced TLR4 protein expression in macrophages and attenuation of the activation of NF-κB, which is involved in the NF-κB cell signalling pathways [259]. Finally, Park et al. have studied the anti-neuroinflammatory properties of surfactin and found that surfactin could significantly inhibit the excessive production of pro-inflammatory mediators in microglial cells [260].

5.6 Immunomodulatory Activity

In addition to its anti-inflammatory activity, surfactin also has many other immune activities. A study by Park and Kim found that surfactin significantly decreased the expression of surface molecules (CD40, CD54, CD80, and MHC-II) of activated macrophages as well as the level of IL-12 [261]. Macrophages treated with surfactin displayed impaired phagocytosis and impaired translocation and activation of NF-κB p65. Moreover, surfactin inhibited the activation of CD4+ T cells, the phosphorylation and degradation of IκB-α, and suppressed the activation of IκB kinase, Akt, c-Jun N-terminal kinases (JNK), and p38 kinase. Surfactin could act as an immunomodulator in autoimmune disease and transplantation, as surfactin impairs the antigen-presenting function of macrophages by inhibiting the expression of MHC-II and costimulatory molecules via suppression of NF-κB, p38, JNK, and Akt.

Surfactin was also shown to trigger an immune response by inducing the maturation of dendritic cells (DCs) in vitro [262]. DCs treated with surfactin showed morphological and phenotypic characteristics of a mature state with high expression of MHC-II, CD40, IL-6, and TNF-α. The induction of DC maturation by surfactin may implicate the NF-κB signalling pathway as surfactin-treated DCs showed an increase in nuclear p65 levels and a decrease in IκB-α levels.

Interestingly, a surfactin produced by *B. amyloliquefaciens* WH1, WH1fungin, has been used as an immunoadjuvant. WH1fungin induces both durable humoral and cellular immune responses, even as strong as Freund's adjuvant by a mixed Th1/Th2 response [263]. WH1fungin was used as an adjuvant with hepatitis B surface antigen and elicited a strong immune response towards the antigen [264]. WH1fungin also suppresses type 1 diabetes mellitus in mice [265].

5.7 Induction of Systemic Resistance in Plants

In addition to their direct antagonistic activities, surfactin, iturin, and, to a lesser extent, fengycin improve plant protection via induction of systemic resistance (ISR). ISR can be conceptualised as a primed state of resistance that renders the plant less sensitive to various pathogen ingress such as microbial pathogens, nematodes, and insects [266–268]. The ISR-state is materialised by the induction of early signalling events of plant defence (i.e. ion fluxes, reactive oxygen species accumulation, and phosphorylation cascade), which ultimately leads to the activation of the salicylic and jasmonic acid responses, the main plant defence response systems [269, 270]. The efficiency of CLPs in inducing ISR is tightly linked to their structures. Indeed, structural variants show differential ISR activities; linear forms of surfactin (hydrolysis of the peptide ring) are much less efficient than canonical ones, and short-length aliphatic chain-bearing surfactin homologues (C12 and C13) are also less efficient than long ones (C14 and C16) to induce ISR in tobacco cells [271, 272]. Therefore, ISR induction has been reported, among others, for surfactin in bean, tomato, tobacco, against *B. cinerea*, in melon against *Podosphaera fusca*, in peanut against *Sclerotium rolfsii*, in lettuce against *R. solani,* and in wheat against *Zymoseptoria tritici* [273–278]. Similarly, iturin family members stimulate plant defences in different pathosystems, such as strawberry against *C. gloeosporioides,* chilli pepper against *P. capsici*, wheat against *Z. tritici*, grapevine against *B. cinerea*, and cotton against *Verticillium dahliae* [279–283]. Finally, fengycin triggers ISR in tomato against *B. cinerea, Plasmopara viticola,* and *Sclerotinia sclerotiorum* [277, 284, 285].

5.8 Biofilm and Motility

CLPs, especially surfactin, play an essential role in biofilm formation, motility, and root colonisation of *Bacillus* cells, which are essential for proper plant protection [66, 216, 286]. Biofilms are usually defined as a communal lifestyle of cells that are encased in an extracellular matrix secreted by the cells. The matrix is composed of polymers (proteins, exopolysaccharides, and DNA) and constitutes a shelter where bacteria are protected from environmental vagaries and competitor ingress [287–289]. For instance, the biofilm matrix prevents *P. chlororaphis* from invading *B. subtilis* 3610 colonies [290]. Beyond its protective role, a biofilm allows cells to coordinate for motile behaviour, allowing migration, and for the production of secondary metabolites, which are crucial for biocontrol [287, 289, 291, 292]. The biofilm thus offers a great fitness advantage for *Bacillus,* and it is not surprising that root-associated *B. velezensis* FZB42 is known to form biofilm structures upon plant roots, such as *Zea mays, Arabidopsis thaliana,* and *Lemna minor* [293]. Coherently, mutants of *B. subtilis* 3610 and *B. subtilis* 6051 impaired biofilm production and exhibited lower biocontrol efficacy of tomato roots towards *R. solanacearum* and

A. thaliana roots towards *P. syringae,* respectively [215, 216]. The mechanism by which surfactin induces biofilm production goes beyond its function as a wetting agent, as it is involved in quorum sensing, which regulates matrix production and motility. The proposed mechanism states that surfactin, when in sufficient amounts, activates Spo0A via potassium leakage, which in turn de-represses biofilm matrix synthesis [103, 104]. Accordingly, mutants of *B. subtilis* 6051, *B. subtilis* OKB120, and *B. atrophaeus* ATCC 9372, deprived of surfactin synthesis, are impaired in biofilm formation [66, 215]. For *B. subtilis* 916, bacillomycin L and surfactin both contribute to biofilm [65]. A recent study reported that surfactin is not required for proper biofilm formation by *B. subtilis* 3610, which suggests that the involvement of CLPs in biofilm formation may be complex and diverse within the *Bacillus* genus [294].

Cell motility is also key for root colonisation, as it allows the bacteria to relocate or colonise new root portions, thereby expanding the biofilm surface. *Bacillus* motility is usually associated with sliding or swarming. Sliding is a passive motility in which cells are pushed away by dividing neighbours, whereas swarming is a coordinated flagellar-dependent motility across surfaces [295]. In either case, cell motility is favoured by surfactants, such as CLPs, as they lower the surface tension and friction between cells [295]. Experimental data show that surfactin-deficient *B. subtilis* mutants were unable to slide and that chemical or genetic complementation could recover the wild-type phenotype [296–299]. Furthermore, mycosubtilin also contributes to the sliding phenotype [181]. Similarly, *B. velezensis* y6 requires all three lipopeptides (surfactin, iturin, and fengycin) for proper cell motility [300]. Similar to biofilm formation, the mechanism by which CLPs enhance motility may go beyond their wetting properties. Indeed, surfactin has been reported to enhance flagellar synthesis, thereby enhancing swarming motility [301].

6 Applications

The global surfactants market size was valued at $41.3 billion in 2019, and is projected to reach $58.5 billion by 2027, registering a compound annual growth rate of 5.3% from 2020 onwards [302]. The biosurfactant market is also increasingly growing because of its more environmentally friendly effects compared to chemical surfactants, owing to their biodegradability.

Owing to the multiple activities of surfactin and mostly to the wide application fields of some of them, surfactin has a broad range of applications [303]. It can replace chemical surfactants, but surfactin also has specific properties and applications. To the best of our knowledge, surfactin is currently being sold in the detergents and cosmetics market.

6.1 Agriculture

Agriculture is a field in which most of the activities of surfactin can be used. It can be used indirectly for the development of beneficial rhizobacteria for plants. The enhancement of root colonisation with the biofilm and swarming activities of surfactin [215, 304, 305] boosts the rhizosphere competence of these bacteria and thus their survival. In addition, surfactin can also be directly used to counter pathogens. This antagonism is due to its antiviral and antibacterial activities [222, 306]. Furthermore, surfactin can be used indirectly against pathogens by triggering ISR in plants. Surfactin has been shown to trigger ISR in tomato, tobacco, and bean plants, but also in rice against certain pathogens [277, 307]. Mycosubtilin and fengycin can also be used as biopesticides with a double mode of action: direct antagonistic activity against several fungal phytopathogens or induction of systemic resistance [308].

6.2 Food

The food sector uses chemical surfactants in an indirect way to clean and treat surfaces in contact with food, but also directly as food additives for solubilising, emulsifying, and foaming capacities. For additive applications, even if *B. subtilis* is generally recognised as safe, surfactin has not yet been used owing to the novel food regulation. Indeed, one of the drawbacks of surfactin is its toxicity above a certain concentration, but because of its high surfactant power, a very low amount would be needed.

As a food additive, surfactin can be used as a food preservative or as a food emulsifier. A study has been performed with the addition of *B. subtilis* surfactant to cookies [309] and bread [310], which enhanced their properties. Huang et al. [311] showed that a mixture of surfactin and polylysine reduced *Salmonella* contamination and that it could be used as an antimicrobial agent. Thus, surfactin can also be added to create a nanoemulsion through its surfactant properties and simultaneously has at the same time an antimicrobial effect. Joe et al. [312] demonstrated that the application of a nanoemulsion of surfactin and sunflower oil to vegetables, chicken, milk, and apple juice protected against three pathogens. However, the main current application of surfactin in the food industry is through its use as a chemical detergent, which will be further discussed.

6.3 Environmental

Surfactin allows the separation of oil from the environment. It can also be used for microbial-enhanced oil recovery in oil reservoirs [313–315]. Surfactin is produced in

high quantities outside the oil reservoir and is then added to extract the component of interest from the soil.

Thus, it can be used for bioremediation and oil recovery from contaminated soils [316, 317]. It can also remove heavy metals from soil [318] and water through the micellar-enhanced ultrafiltration technique [319].

6.4 Pharmaceutical

Surfactin can be used in the pharmaceutical field because of its many features. First, its membrane-disrupting activity leads to antiviral [238, 239], antibacterial, and antimycoplasmic [320] applications. Immunomodulatory activity could lead to the use of surfactin in the treatment of allergies, diabetes, arthritis, and autoimmune diseases, and help for transplantations [321]. The anticancer activity of surfactin has been demonstrated against multiple cancer types: Ehrlich ascites, breast, colon, leukaemia, hepatocellular, cervical, oral epidermoid, pancreatic, and rat melanoma [322]. Lastly, Chen et al. [321] have shown that surfactin has an effect on lipase and could therefore be used as an anti-obesity drug.

The major drawback of using surfactin in the pharmaceutical field is its haemolytic activity. However, it was shown that the concentration needed is lower than the one showing haemolytic behaviour: the haemolytic activity appears at 40–60 μM [232], whereas the anticancer activity is shown at 30 μM [243]. Another possibility to counter toxicity is to modify the chemical structure. Indeed, as mentioned before, linear surfactin is not haemolytic and can protect red blood cells from other surfactants.

The antibacterial activity of surfactin against *Legionella* opens its potential use for the cleaning of facilities with stagnant water [222].

6.5 Detergent

The detergent application is the one in which the change of chemical surfactants by biosurfactants is most interesting from an environmental point of view since surfactin itself brings no new activity. However, its higher biodegradability is very important for the future of this sector. As previously mentioned, surfactin has antiadhesive activities that can hinder microbial and biofilm development. In the food industry, where microbial development is a risk to public health and product deterioration, the development of biofilms is of great concern. Surfactin was thus used by do Valle Gomes and Nitschke [323] to remove food pathogen biofilms. Surfactin inhibited biofilm formation by pathogenic organisms by preventing them from adhering to solid surfaces, infection sites, or catheters. These pathogenic organisms include *Salmonella enterica, S. enterica* serovar Typhimurium, *E. coli*,

and *Proteus mirabilis.* Surfactin at concentrations between 5 and 50 mg/L can inhibit biofilm formation by *Salmonella* on catheters [324].

The surfactant properties of surfactin can also be used as laundry detergents. A mix of biosurfactants produced by a *B. subtilis* strain producing mainly surfactin, added to a chemical detergent, showed a high wash quality [325]. Furthermore, surfactin does not inhibit the activity of subtilisin, a *Bacillus* protease often used in laundry formulations [326].

6.6 Cosmetics

Owing to its antibacterial, emulsifying, and deterging activities, surfactin has a broad range of applications in cosmetics, such as cleansing products and external skin preparations, and many patents mention it in their cosmetic composition [327]. Surfactin has been used as a carrier in a mixture to encapsulate active substances. These carriers enable the transport of substances deep into the skin, stopping at the dermis border and reducing the discolouration, vascular lesions, and depth of the wrinkles on the tested skin [328]. Moreover, a study by Yan et al. demonstrated that surfactin has effects on wound healing, angiogenesis, cell migration, inflammatory response, and scar formation, demonstrating the potential application of surfactin as a wound-healing drug [329]. Finally, patents have been filed for the use of surfactin in cosmetics, demonstrating anti-aging and anti-wrinkle activities and increasing skin penetration of cosmetic products (skin penetration agent), highlighting the broad applications of surfactin in this field [330, 331].

7 Conclusion

During the last 30 years, the literature on lipopeptides from *Bacillus* has exponentially increased, demonstrating the interest of these compounds. Their remarkable structural biodiversity offers many bioactive compounds with many applications in different sectors. The progress in their production optimisation has rendered their commercial development possible. In addition, the modulatory mode of their biosynthesis and the deep knowledge of how they function should lead to the build-up, by synthetic biology, of a novel generation of novel compounds with increased beneficial properties and decreased cytotoxicity.

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Achieving Commercial Applications for Microbial Biosurfactants

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Contents

Abstract There are numerous biosurfactant producing strains and products reported annually most of which aspiring to potential future industrial and environmental applications. Only a few of these compounds have reached commercial applications due to many impediments to large-scale production and applications. We investigate some of the important criteria and investigations required to achieve such future commercial application.

Keywords Commercial exploitation, Downstream processing, Microbial biosurfactants, Production cost, Safety

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1 Introduction

The literature is replete with numerous papers reporting the production of biosurfactants from already well-characterised bacteria, from newly isolated bacteria, new biosurfactant compounds, the use of recycled waste feedstocks and using new fermentation strategies. Many of these reports contain the aspiration for their new product or process to find a place in the panoply of commercial products [1, 2]. Unfortunately, these aspirations have only been achieved for a small proportion of the biosurfactants that have been extensively researched [3, 4]. In this chapter we will examine what are some of the essential criteria that need to be met for a new biosurfactant to be adopted and incorporated into a commercial product and more importantly what are the impediments to its uptake. Using examples of microbial biosurfactants already in use and those that have not yet found any use we will try to identify the key critical points that may be amenable to future experimentation and development to increase the range of biosurfactants used.

There are three major considerations that drive whether a new biosurfactant is likely to be suitable for a particular commercial application these are: *SAFETY, EFFICACY* and *COST*. In addition to these major considerations there are a number of other subsidiary factors which will also be taken into account before a final commitment can be made regarding the use of a particular biosurfactant in a product or formulation [5].

2 Safety

In general, microbial biosurfactants have not faced major issues of safety; however, the type of organism producing the biosurfactant is a critical factor in the issues that do exist. Some of the most thoroughly investigated glycolipid biosurfactants, e.g. sophorolipids and mannosylerythritol lipids (MELs) are produced by yeasts and yeast-like fungi and although some close relatives of the producer organisms may be pathogenic to plants and indeed humans there has been no suggestion of the production of any toxins produced by these organisms that can contaminate the biosurfactants produced. The situation is however rather different for the biosurfactants produced by some of the bacterial species, the best example of which are the rhamnolipids produced by *Pseudomonas aeruginosa.* Extensive research, over many years, has been carried out using various strains of this Gram-negative bacterium despite the fact that it is a category II human pathogen.

P. aeruginosa is an opportunistic pathogen important in wound infections and a major complicating lung infection in cystic fibrosis patients and is responsible for substantial nosocomial mortality [6]. The organism produces a range of virulence factors such as elastase and pyocyanin and in addition has an LPS endotoxin originating from the cell membrane structure of the cells. The production of endotoxin is a likely outcome from any process using a Gram-negative producer

organism and should always be borne in mind [7]. The endotoxins produced by Gram-negative bacteria are highly bioactive even at very low concentrations and while they may be tolerated in some applications, food and pharmaceutical products require special care. Due to the infection and toxicity risks associated with the use of *P. aeruginosa* companies in Europe have been very unwilling to consider the use of rhamnolipids from this organism for use in commercial products. Interestingly in the USA large-scale fermentation production of *P. aeruginosa* rhamnolipids does seem to take place even in proximity to production of other food product components.

Due to the safety concerns with *P. aeruginosa* researchers have been seeking alternative means of producing rhamnolipids from other non-pathogenic organisms. This approach has taken two routes: (1) Bioprospecting for new bacterial species, (2) The metabolic engineering of related bacteria such as *Pseudomonas putida* to express the rhamnolipid synthetic genes of *P. aeruginosa.* The first route has resulted in the discovery of new organisms such as *Burkholderia thailandensis* which uses a similar synthetic pathway to *P. aeruginosa* but with non-homologous genes [8]. In this case the rhamnolipids have slightly longer lipid chains which give different physicochemical characteristics to the biosurfactant. Unfortunately the yields of rhamnolipids from *B. thailandensis* do not yet make it an attractive commercial producer organism even when the synthetic pathway for the storage material polyhydroxyalkanoate (PHA) is knocked out to direct more metabolites towards rhamnolipid synthesis and the quorum sensing system is manipulated [9, 10]. Other recently reported strains of non-pathogenic Pseudomonas and Marinobacteria have been reported to produce low concentrations of rhamnolipids and remain under investigations [11, 12]. The second strategy has been pursued successfully by EVONIK who have metabolically engineered the rhamnolipid genes into a non-pathogenic producer organism and have at the same time overcome the tight genetic regulation imposed by quorum sensing in *P. aeruginosa* to give much higher product yields as was reported by the Evonik and Unilever collaboration for large-scale production of the world's first "green" biosurfactant (see Ref. [13]).

3 Efficacy

Microbial biosurfactants have two possible broad routes to commercialisation, first as replacements, complete or partial, for chemical surfactants in existing product formulations and second as components of entirely new products exploiting specific characteristics of the biosurfactant molecules. If we consider the first option, we need to be aware that there are different forms of surfactants that have specific applications in particular products. These different forms are characterised by differences in the charge carried on the hydrophilic end of the molecule, thus there are neutral surfactants without any charge, anionic surfactants with a positive charge, cationic surfactants with a negative charge and zwitterion surfactants (amphoteric) carrying both a negative and positive charge. Examples of commonly used chemical surfactants in these categories are given in Table 1.

Table 1 Commonly used chemical surfactants and their applications

General Structure	Common/ Trade Name	Structure/Name	Applications
Nonionic surfactants	Triton™X-100	Polyoxyethylene glycol octylphenol ethers	Wetting agent – coating
	Nonoxynol-9	Polyoxyethylene glycol alkylphenol ethers	Spermicide
	Polysorbate	Polyoxyethylene glycol sorbitan alkyl esters	Food ingredient
	Span®	Sorbitan alkyl esters	Polishes, cleaners, fragrance carriers
Anionic surfactants	Calsoft®	Linear alkylbenzene sulfonates	Laundry detergents
	Texapon®	Sodium lauryl ether sulphate	Shampoos, bath products
	N/A	Sodium stearate	Hand soap
Cationic surfactants	CPC	Cetylpyridinium chloride	Antimicrobials
	CTAB	Cetyl trimethylammonium bromide	Antimicrobials
Amphoteric surfactants	Sultaines	Sulphonates	Various
	Betaines	Cocamidopropyl betaine	Various, e.g., fast dry paints

A cursory examination of the list of ingredients in common household laundry, cleaning and personal care products reveals that these different products employ specific types of surfactants depending on the type of application. Where detergency is the prime requirement, as in non-biological laundry products, anionic surfactants are the surfactants of choice, with up to 30% of the formulation comprising alkylbenzene sulfonates. In the equivalent biological laundry detergent products, the surfactant components of the formulation are selected to be compatible with the lipases and proteases that are incorporated to aid the cleaning process. In contrast laundry fabric softeners contain 5–15% cationic surfactants while we find betaines as important constituents of personal care products such as hand sanitisers and shampoos, due to their antimicrobial activity, formulated with anionic surfactants such as SLS. In other home-care products such as surface cleaners the products contain low concentrations of amphiphilic and non-ionic surfactants while dishwashing preparations are a mixture of anionic and non-ionic surfactants. What is immediately obvious from scrutiny of the above information is that the surfactant constituents of these commercial products are carefully chosen to carry out specific functions. It is equally clear that we cannot expect to simply replace the chemical surfactants in products directly and completely with microbial biosurfactants, particularly since most biosurfactants are either anionic or amphiphilic with only a few containing amine groups being cationic.

For a biosurfactant to be acceptable as a complete or partial replacement for an existing chemical surfactant in a commercial product the performance of the biosurfactant must be at least as good, or preferably better, than the compound it is replacing. We will deal with the issue of cost in the next section of this article. Also,

since the range of types of biosurfactant is more limited than the chemical versions the range of potential applications is also more limited. Various characteristics of the biosurfactants are relevant to the potential applications e.g. certain products like dishwasher preparations need to have the capability to foam since customers link foaming with cleaning. Obviously, detergency is a key capability for a biosurfactant in a laundry product, however, they often compete poorly with chemical surfactants in this area.

3.1 Exploitation of Unique Biosurfactant Properties

Rather than viewing biosurfactants simply as replacements for existing surfactants in use an alternative approach is to consider possible unique properties of these molecules as a different route to exploitation. An important ability of surfactants is emulsification which allows the formation of emulsions of oil in water or water in oil. One of the biological functions of biosurfactants in microbes is believed to be the conversion of oily substrates into an accessible form for the microbial cells. In general, the low molecular weight biosurfactants such as the glycolipids are not highly efficient emulsifiers while the high molecular weight biosurfactants such as lipopolysaccharides and lipopeptides can produce stable and long-lasting emulsions. Potential applications for microbial biosurfactants in this area include the formulation of pharmaceutical and personal care creams, particularly with the increasing concerns about some of the detrimental effects of the chemical components that have been used previously like parabens [14, 15]. Unfortunately, some of these applications are not entirely straightforward since some of the purified biosurfactant preparations, e.g. rhamnolipids are coloured and have an odour which makes their use in cosmetic products extremely problematic.

One of the major requirements in the food industry is for emulsifiers that are capable of maintaining stable emulsions for extended periods to ensure suitable shelf life for the product. The idea that microbial biosurfactants are natural ‘green’ products is inviting as a marketing strategy, while they can also be considered as sustainable through their production by fermentation processes using renewable substrates [16]. Many ‘natural’ and modified emulsifiers are currently used such as lecithins, which are mixtures of phospholipids, esters of monoglycerides of fatty acids created with acetic, citric, lactic or tartaric acid, mono and diglycerides of fatty acids, xanthan, polysorbate, and carrageenan. There have been suggestions that microbial biosurfactants/emulsifiers ranging from the low molecular weight sophorolipids to the high molecular weight lipopeptides could have applications in the food industry (reviewed by [17]), however, although many of these microbial products do have emulsifying activity the longevity and stability of the emulsions do not always meet the requirements necessary for commercial exploitation. Also the safety issues are clearly paramount for food applications which places constraints on the use of producer organisms that are not Group I; generally regarded as safe (GRAS) microorganisms and while some of the producer microorganisms,

particularly the yeasts, may have few issues, testing and certification of products from many of the bacterial producers would be time consuming and costly and probably not worthwhile unless the advantages were significant. However, potential application of high molecular weight biosurfactants as gelling agents does have some possibilities. The example of the effort and cost that was required to achieve acceptance of the fungal single-cell mycoprotein QUORN indicates the potential difficulties in this area to [18].

A more promising route for further commercialisation of biosurfactants is probably through exploitation of their bioactivities. There is an increasing list of antimicrobial, anticancer and other biological activities being ascribed to microbial biosurfactants. The potential to make use of these specific activities does open the door for a new range of applications. There are however some hurdles which need to be overcome first. Many of the microbial biosurfactants are not produced by the organisms as a single type of molecule but as a mixture of different congeners of related molecules. For example, the rhamnolipids produced by *P. aeruginosa* and other bacteria are produced as either mono- or dirhamnolipids with a range of different lipid chain lengths [19, 20] while the sophorolipids of the yeast *Starmerella bombicola* are produced in acidic and lactonic forms [21]. Because the congeners are very similar in size and chemical composition post-production separation is difficult and while some metabolic engineering is possible to reduce the product diversity it is not always possible to direct the synthesis exactly as desired. It has already been shown that different congeners of a biosurfactant may have different and even opposing activities [22] which means that any claims for specific bioactivity must be carried out using highly purified single congeners, which would in any case be necessary for any quality controlled biomedical application. Major bioactivities of microbial biosurfactants are their biofilm disruption potential and their direct biocidal and biostatic capabilities [23–28]. The biofilm disruption capability has application particularly in surface cleaning preparations while the anti-bacterial effects could be exploited in oral health products such as toothpastes and mouthwashes and in skin cleansing and treatment preparations [29]. The critical aspect with biosurfactant use in products for skin application is how selective is the biocidal activity since the maintenance of a balanced skin microbiome is essential for health and a complete non-selective biocidal activity would be undesirable.

In contrast to the glycolipid family of biosurfactants there is a group of lipopeptide biosurfactants such as the three families of cyclic compounds surfactin, iturin and fengycin produced by Gram positive members of the genus *Bacillus*. Each family contains variants with the same peptide structure but with residues at different positions and different length fatty acid chains. Surfactin, for example, is produced in four different congeners by *Bacillus subtilis* with a common seven loop amino acid cyclic peptide linked to a long chain fatty acid (C10–C13) [30]. Surfactin is highly effective at reducing surface tension giving a surface tension value of 27 mN/m at a concentration of 20 μM in water and has been demonstrated to have a number of bioactivities including anti-bacterial, anti-viral and anti-fungal. The potential applications for biosurfactants of this type therefore appear promising, however, the critical problem for economic exploitation resides with the yield of these

products. Yields from fermentation systems using expensive complex media tend to be in the 2–3 g/L range making any production process prohibitively expensive [30].

Lipopeptide biosurfactants are also produced by other bacterial groups including *Pseudomonas* [31] where they have been classified into four major groups: viscosin, amphisin, tolaasin and syringomycin [32]. Once again there is structural diversity in the molecules produced and they have a wide range of different bioactivities but the problems of developing a production system to produce economic quantities of sufficiently pure compounds using naturally occurring strains seem remote. A metabolic engineering approach could however provide a route to future use of these types of microbial biosurfactants in niche products exploiting their specific bioactivities.

4 Cost

The third major consideration after safety and efficacy for the exploitation of biosurfactants is cost. The cost of the final biosurfactant product that can be used in any commercial formulation will depend on a number of specific factors. The most important and probably the one which provides the greatest current hurdle for most biosurfactants to reach the market is the yield of the product in the fermentation process. Most natural bacterial producers only achieve yields of tens of grams per litre. This problem has been overcome by EVONIK through the metabolic engineering of rhamnolipid synthetic genes into a new host organism. The best producer organisms are by far the yeast and fungal strains producing sophorolipids and MELs where yields are in the range of hundreds of grams per litre by naturally occurring strains and this is certainly the reason why these are predominant in the market [33].

We can gain some idea of the target cost necessary to strive towards for effective commercialisation by re-examining the consumer products currently on the market. For example, laundry detergent costs €4–5/L retail and contains up to 30% surfactant, which immediately dictates a very low cost for the surfactant component. Another more recent proposed application include potential use in sanitising cleaning and antimicrobial formulations that can be effective in combating COVID019 and similar future microbial threats [34]. We have to consider that these microbial biosurfactants are secondary metabolites and are generally produced towards the end of a batch fermentation implying long fermentation times with high energy costs. Some of the substrates that can be used for the fermentations are relatively cheap, however, the suggestion is frequently made in publications that waste materials can be used to reduce the cost, but unless the waste is available at sufficient scale, with consistent composition this may not be a viable option. Waste materials are also probably of complex mixed composition increasing the difficulties of producing a pure final product in the downstream processing system and once waste materials have some use it would mandate value.

Downstream processing of BSs plays a vital role in their purity, production costs and potential applications. The most commonly used solvent extraction processes

have many disadvantages when products are to be used in food and pharmaceutical applications. As the demand for biosurfactants increases, developing new downstream processes or fine-tuning of existing methods becomes more important. One such approach could be the utilisation of the micelle behaviour of biosurfactants at concentrations higher than their CMC allowing phase-separation from the culture broth and precipitation which allows easier isolation from fermentation broths and further purification by exclusion filtration. The use of ultrafiltration of BSs where micelles can lead to an increase in the molecular size of the BSs improving membranes retention and/or precipitation by centrifugation is also important [35]. Another important potential downstream process is through using Supercritical CO_2 ($ScCO_2$) as a solvent to separate and fractionate biosurfactants from their production medium. This converts current environmentally and economically costly processes, based on solvent extraction processes to a more economic and environmentally sustainable process with practically zero waste, since the CO_2 can be recycled, and with the added advantage of being able to separate the main congeners of these biosurfactants [36].

5 Flexibility of Biosurfactants

There are available to manufacturing companies a wide range of different chemical surfactants that can be combined in specific formulations to achieve the desired final product. In contrast the range of different biosurfactants is more limited (Table 2) and more importantly it is not straightforward to make subtle changes to their structure. The whole situation is also made more complicated by the fact that we are not able to accurately predict how changing the structure of a biologically produced surfactant molecule would alter its physicochemical or biological characteristics. Some attempts have been made to create ‘new to nature’ biosurfactants, most notably by van Bogaert and her co-workers using *Starmerella bombicola* produced sophorolipids [37]. How far this approach can be further exploited remains to be seen.

Table 2 The main types of biosurfactants, producer organisms and their general application

Biosurfactant		Producing microorganisms	General applications
Structure	Types		
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas sp.* *Burkholderia thailandensis* *Other Burkholderia sp.*	Enhancing hydrocarbons bioavailability, biodegradation, dispersion, emulsification Enhanced oil recovery Antimicrobial and biomedical
	Trehalolipids	*Rhodococcus erythropolis, Arthrobacter sp.* *Nocardia erythropolis* *Mycobacterium tuberculosis* *Corynebacterium sp.*	Enhancement of the bioavailability of hydrocarbons
	Sophorolipids	*Starmerella bombicola* *Candida antarctica, C. batistae* *C. apicola,* *C. riodocensis,* *C. stellata,* *C. bogoriensis* *C. lipolytica*	Enhancing bioavailability of hydrophobic compounds, biodegradation, dispersion, emulsification of hydrocarbons Laundry detergent agents antimicrobial and biomedical
	Mannosylerythritol lipids	*Ustilago zeae,* *U. maydis* *Pseudozyma fusiformata* *Sympodiomycopsis paphiopedili*	Antimicrobial, immunological and neurological biomedical application
	Cellobiose lipids	*Ustilago maydis* *Cryptococcus humicola* *Pseudozyma aphidis* *Pseudozyma. hubeiensis*	Detergent, antimicrobial, antifungal and biomedical applications
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery
	Spiculisporic acid	*Penicillium spiculisporum* *Corynebacterium lepus* *Arthrobacter paraffineus* *Talaromyces trachyspermus*	Dispersion, emulsification, organogels formation, superfine microcapsules production (vesicles or liposomes) Heavy metal sequestrants

(continued)

Table 2 (continued)

Biosurfactant		Producing microorganisms	General applications
Structure	Types		
		Nocardia erythropolis	
	Phosphatidylethanolamine	*Acinetobacter sp., Rhodococcus erythropolis*	Pharmacological application Food products
Lipopeptides	Surfactin Iturin Fengycin	*Bacillus subtilis Bacillus amyloliquefaciens Bacillus pumilus*	Enhanced biodegradation of hydrocarbons and chlorinated pesticides; heavy metal removal. Enhanced phytoextraction. Biomedical and cosmetic uses
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery
	Rhodofactin	*Rhodococcus sp.*	Bioremediation
	Viscosin, amphisin, tolaasin, syringomycin	*Pseudomonas fluorescens Pseudomonas spp. Leuconostoc mesenteroides*	Bioremediation and biomedical applications
	Subtilisin	*Bacillus subtilis*	Antimicrobial properties
Polymeric biosurfactants	Emulsan	*Acinetobacter calcoaceticus RAG-1*	Stabilisation of hydrocarbon-in-water emulsions Bioemulsifiers
	Alasan	*Acinetobacter radioresistens*	
	Biodispersant	*Acinetobacter calcoaceticus*	
	Liposan	*Candida lipolytica*	
	Mannoprotein	*Saccharomyces cerevisiae*	
Particulate biosurfactants	Whole cells vesicles and Fimbriae	*Acinetobacter calcoaceticus Pseudomonas marginalis P. maltophilia, Cyanobacteria*	Bioflocculants and biodegradation

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Process Development in Biosurfactant Production

Robert W. M. Pott and Janis Von Johannides

Contents

Abstract Biosurfactants encompass a number of structurally and chemically diverse compounds, all of which demonstrate surface active properties. The potential and current application of these compounds ranges from enhanced oil recovery, through detergents, emulsifiers in foods, antifungal agents, antibiotics, and even to uses in the minerals processing industry. And while the market demand for these products is growing, the industry still is significantly smaller than the synthetic surfactants market. Part of the reason for this is that biosurfactants are currently comparatively expensive to produce. This article reviews the process consideration

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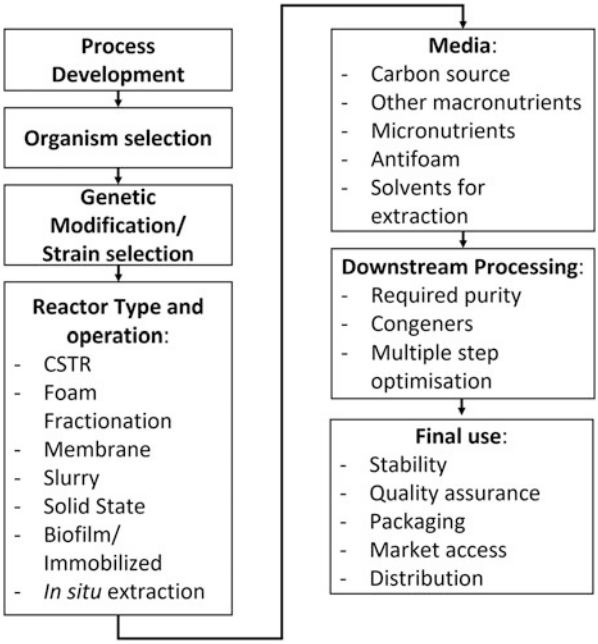
steps required to develop a biosurfactant bioprocess (organism selection and modification, reactor type and operation, media, downstream processing, and final use) and considers the state of the art of each process step, with an eye to considering the overall process development, and establishment of both technically and economically viable routes to production.

Graphical Abstract



Keywords Bioreactor choice, Biosurfactants, Industry review, Process choice, Process development

1 Introduction

Biosurfactants are biologically produced molecules which contain both hydrophobic and hydrophilic moieties. Such molecules exhibit a variety of properties, the most pertinent of which is their ability to change the interfacial tension between phases of the solution they are in – they are SUrface ACTive AgeNTS. This surface activity can then be utilised in a number of applications; from use as detergents, in cosmetics, in enhanced oil recovery, as flotation agents, in bioremediation, or as a foaming agent, to name a few [1]. Table 1 gives a list of non-exhaustive list of applications noted in the literature, along with the biosurfactants used for each (where identified –

Table 1 Examples of applications of biosurfactants

Biosurfactant applications	Biosurfactant used		References
Bioremediation, soil washing and oil spill remediation	Rhamnolipids		[2–5]
	Cell cultures		[6–10]
Enhanced oil recovery and processing	Indigenous bacteria (i.e. a variety of biosurfactants)		[11–17]
	Rhamnolipids		[17–21]
	Lipopeptides		[22–26]
Antibiotic/antimicrobial	Lipopeptides	Surfactin	[27–31]
		Daptomycin	[32–34]
	Glycolipids	Rhamnolipids	[3, 35]
		Sophorolipids	[36–39]
		Mannosylerythritol lipids	[40, 41]
Antifungal	Lipopeptides	Cell cultures	[42–44]
		Iturin	[45–47]
		Fengycin	[48–50]
	Glycolipids		[51, 52]
Flotation	Multiple biosurfactants		[53–57]
Metal ion chelation	Multiple biosurfactants		[58–61]
Detergents	Glycolipids, lipopeptides		[62–64]
Emulsifiers	Multiple biosurfactants		[65–68]
Cosmetics	MELs, glycolipids		[69–71]

alternatively the organism used). Some of these applications are more mature than others, for instance there is significant variability in the results described by literature in bioremediation, whereas use of biosurfactants in enhanced oil recovery is widespread. Nonetheless, the list of applications is long and varied: the usefulness of biosurfactants is recommended by their versatility.

Biosurfactants are produced by a number of living organisms – from microorganisms (bacteria, yeast, fungi) to plants and animals. However, baring saponified fatty acids (a chemical modification of biological triglycerides) and saponins (sourced from plant material) the overwhelming majority of biosurfactant work has been conducted in the microbial sphere [72]. This review will therefore focus on microbially produced biosurfactants.

In their general form biosurfactants usually take the shape of a hydrophobic ‘tail’ (so named since it is commonly composed of a linear alkyl or alkaryl, usually derived from a fatty acid) and hydrophilic ‘head’ (which can be comprised of a number of species: amino acids or polypeptides, carbohydrates (of various types), phosphate, carboxylic acid or alcohol). Table 2 gives a non-exhaustive list of biosurfactants, in their generalised categories, as well as the organisms found to produce each.

The biological pathways responsible for the production of these compounds are frequently promiscuous, and so produce a range of similar, but compositionally

Table 2 Categories and classes of lipopeptides and their producing organisms

Biosurfactant type	Biosurfactant	Organism which commonly produces
Glycolipids	Rhamnolipids	*Pseudomonas sp., Serratia rubidae, Marinobacter sp.*
	Sophorolipids	*Candida apicola, Rhodotorula bogoriensis, Wickerhamiella domercqiae, Starmerella bombicola, Torulopsis bombicola*
	Trehalolipids	*Rhodococcus sp., Arthrobacter sp., Nocardia erythropolis, Mycobacterium sp., Corynebacterium sp.,*
	Cellobiose lipids	*Pseudozyma sp., Ustilago maydis, Cryptococcus humicola*
	Mannosylerythritol lipids	*Pseudozyma sp., Ustilago sp., Schizonella melanogramma, Candida Antarctica, Kurtzmanomyces sp.*
	Polyol lipids	*Aureobasidium pullulans, Rhodotorula sp.*
Lipopeptides	Surfactin	*Bacillus sp.*
	Bacillomycin (Iturins)	*Bacillus sp.*
	Fengycin	*Bacillus sp.*
	Daptomycin	*Streptomyces roseosporus*
	Viscosin	*Pseudomonas fluorescens*
	Peptide lipids	*Bacillus sp., Pseudomonas sp., Rhodobacter sp.,*
	Serrawettin	*Serrati sp.*
	Echinocandins	*Papularia sphaerosperma, Glarea lozoyensis, Aspergillus sp., Actinoplanes utahensis, Zalerion arboricola, Emericella rugulosa, Tolypocladium parasiticum, Candida fermentati*
	Lichenysin	*Bacillus licheniformis*
	Pontifactin	*Pontibacter korlensis*
	Unidentified	*Paenibacillus sp.*
Polymerics	Emulsan	*Acinetobacter calcoaceticus*
	Biodispersant	*Acinetobacter calcoaceticus*
	Alasan	*Acinetobacter radioresistens*
	Liposan	*Candida lipolytica, Yarrowia lipolytica*
Phospholipids	Phospholipids	Essentially all
Nucleolipids	Nucleolipids	Marine sponges
Hydrophobins	Hydrophobin	*Trichoderma reesei, Schizophyllum commune*

distinct, products known as congeners [73]. This variability represents both a significant difficulty (in that purification and separation of congeners is complex and expensive) and a significant opportunity: subtly different congeners can have different properties [74]. A group of compounds with variable properties is a rich seam from which to mine activity for novel applications.

Market analysis for 'natural surfactants' (a category which includes both microbially produced biosurfactants, and other biologically based surfactants) was approximately US$14.3 billion in 2018 [75], and while it expected to grow at a

healthy compound annual growth rate of 4.9%, its size is still dwarfed by that of traditional, synthetic surfactants at approximately US$45 billion in 2018. Of course, these market indicators are just that – indicators – and do not give particular insight into the technical development of novel biosurfactants. Nonetheless, the attraction of the market towards biosurfactants lies, partly, in their potentially lower environmental impact. By their nature they are biodegradable, and so do not persist in nature long. Further, they can be produced from renewable resources and do not rely on fossil resources for their production. Nonetheless, a significant shift in industry would be needed for biosurfactants to eclipse their synthetic cousins. This shift is fundamentally driven by the economics of production and consumption. Of course, no product has zero impact, and a proper analysis of environmental benefits and limitations of each process would be needed to make sensible comparisons. Tools such as life-cycle analysis can assist with this, although comparatively few have been performed on biosurfactants or their comparison to synthetic surfactants [76–78].

Techno-economic analyses [79, 80] of biosurfactant production processes are unfortunately fairly rare. However, there are industrial players in this field which operate profitably on the production of biosurfactants (see the excellent review by Geetha et al. [17] for, amongst other information, details of which companies currently operate). Of work that is available in the literature, much focusses on rhamnolipids, or sophorolipids, for the most part since the organisms which produce these biosurfactants can produce them at significant concentrations. However, it is not sufficient to simply relegate all low-concentration products to non-economic viability; it is important to consider the whole of a potential bioprocess. Currently, biosurfactants are significantly more expensive to produce than synthetic surfactants [81], and so much progress is needed in process intensification and cost reduction. In order to achieve this reduction in cost, it is sensible to break down a process into constituent stages, and consider the state of the art of each in turn. This review therefore aims to break down the development of a biosurfactant-producing process into a series of choice-steps, to examine what has been done in the literature within each space, and to highlight where areas of improvement might be possible.

2 Process Considerations

In the development of a bioprocess to produce biosurfactants, many choices are made which affect the trajectory and techno-economic viability of the process. Presented here is a non-exhaustive look at some of the considerations when developing a process, with an eye to understanding the state of the art in each regard, and with some notation of potential areas for improvement. Figure 1 presents the sections schematically, and the following paragraphs will deal with each point in turn.

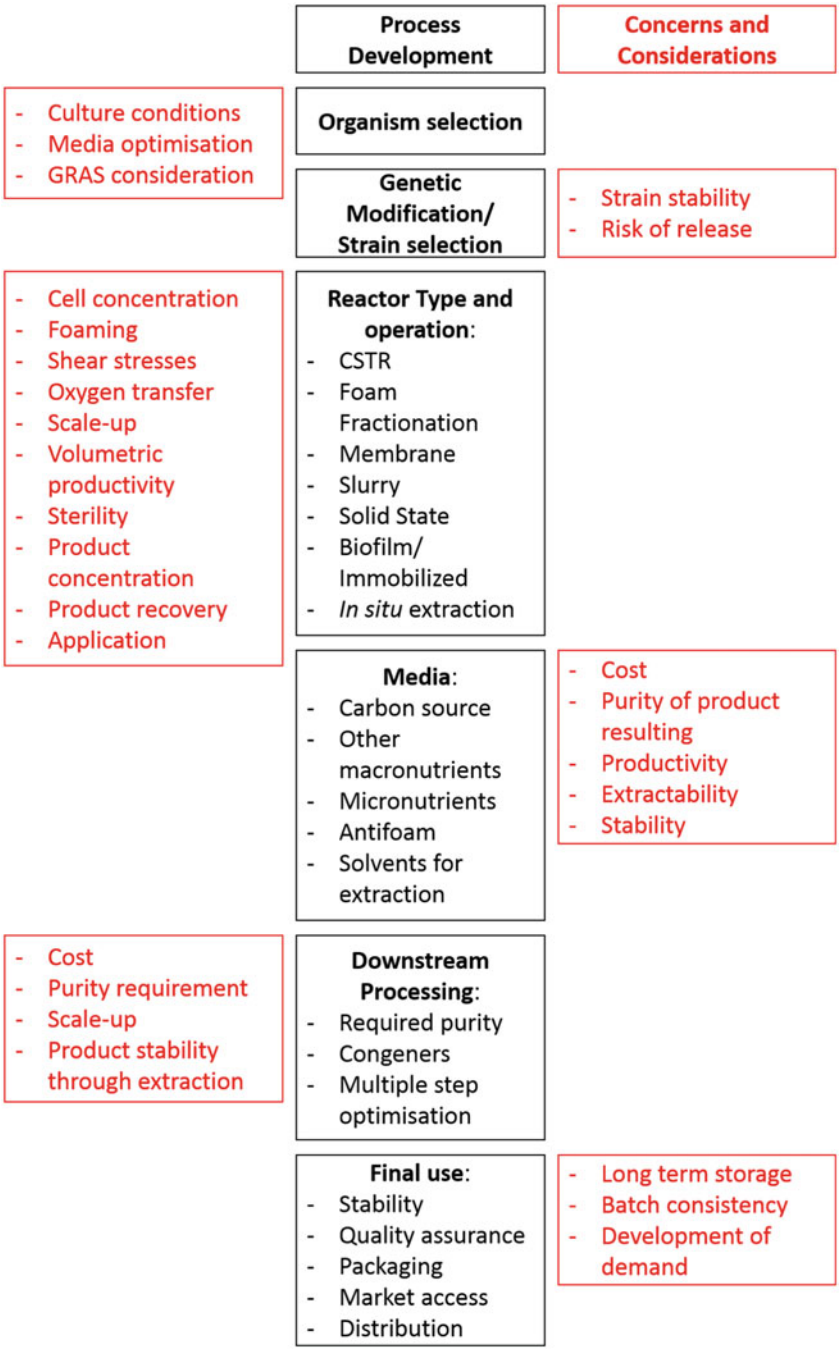


Fig. 1 Schematic flow diagram of points of consideration in the development of a biosurfactant-producing process

3 Organism Selection

A significant portion of the biosurfactant literature deals with isolation and identification of biosurfactant-producing organisms. Strains of interest first need to be harvested from source, oftentimes referred to as bioprospecting, and then isolated using various culture techniques. Subculturing is often accompanied by enrichment with the target for bioremediation (such as hydrocarbon pollutants) across a dilution series. Strains that efficiently utilise the compound of interest, preferentially as the sole carbon and energy source, and which attain the highest cell density at a certain OD, are then subjected to a battery of assessments, including Gram-staining analyses, motility, shape (e.g. rod-shaped, coccoid, bacilli), catalase (or other enzyme) sensitivity, and other tests, if necessary. Isolates displaying higher levels of biosurfactant production and generally also emulsification properties (or oil displacement), depending on end use, as well as an acceptably high growth rate, will undoubtedly generate attention for bioremediation, biocontrol or pharmaceutical application. Genetic identification is done using 16S rDNA sequencing analysis and thereafter a phylogenetic tree is useful to detect evolutionary relationships between species.

Biosurfactant-producing organisms might also be selected based on their potential to benefit or facilitate specific purposes, such as an isolate that demonstrates ability to degrade a metal or pesticide known to contaminate marine reserves [82]. Countless papers have been published identifying new organisms producing a variety of known and novel biosurfactants; however, there seems to be a dearth of information characterising production pathways or investigations into why some microbes manufacture different congener profiles or homologue analogues under certain conditions. Of course, the bioprospectors should not stop – you can't find something interesting if you're not looking, but once identified, the organism should be sequenced, the known pathways for biosurfactant production looked for in the sequence, and the surfactant analysed by GC-MS, if possible, or using other advanced analytical techniques. Simply showing surface tension changes is more of a high school experiment; the road to commercial relevance is still littered with question marks.

4 Genetic Modification/Strain Selection

Seyedsayamdost, in his 2019 review of bacterial secondary metabolism, spoke about 'biosynthetically gifted microbes', to which many biosurfactant-producing organisms, such as *Bacillus subtilis*, lay claim. The Princeton paper pointed out that the research community is yet to elucidate the secondary metabolome of these microbial powerhouses [83]. In Jimoh and Lin, the authors, too, concede that existing research inadequately illustrates the molecular profile of biosurfactants [84]. These secondary metabolites, which predominate the late exponential and stationary growth phases,

have much to do with enabling a microbe's exogenous experience. Exogenous variables could be chemical (nutrients, pesticides, metals, intra-/interspecies communication signals, pH changes, aeration, oxidative stress) or physical (temperature changes or heat shock, agitation, exposure to radiation or UV light), which in turn induce epigenetic changes to the organism's genetic sequence. It's imperative that we gain some perspective on the different feedback loops involved in biosurfactant metabolism – some level of molecular characterisation will go a long way in elucidating downstream output variance.

A review published in Biotechnology Journal in 2017 elegantly sets out numerous heterologous production strategies used in rhamnolipid and surfactin titre enhancement, differentiating between approaches taken using non-pathogenic Pseudomonas and other species such as *Burkholderia* and *E. coli*. The paper touches on quorum sensing (QS) networks used by various bacterial strains and how the QS cascade could be triggered by either paracrine signalling, in the case of *Bacillus*, or autocrine communication markers [85]. The QS molecule, ComX, is thought to be a key component in boosting surfactin yield in *Bacillus* sp., however, an understanding of the molecular network that underplays productivity is essentially a mystery. Overcoming knowledge gaps in these regulatory induction pathways would bode well for commercialisation of biosurfactants. Mannosylerythritol lipids (MELs) and sophorolipids (SLs) are suited to bulk production using wild-type strains and their progeny (rhamnolipid development is thought to be once again progressing in this direction, although with recombinant strains); however, metabolic engineering approaches in augmenting yields would significantly reduce costs and molecular biology tools are appealing as an aid in amplification of target metabolites. Interestingly, an in silico study found that metabolic modelling and knockout prediction of the precursor leucine intensified surfactin production 20-fold [85].

Jimoh and Lin note that recombinant strains exhibit improved product outcomes (more uniform output in terms of congener profiles) as well as effecting reduction in the number of spurious (redundant) proteins, thus simplifying purification and recovery protocols [84]. Engineered *Saccharomyces cerevisiae,* as a GRAS status organism, has been reported to produce monorhamnolipid utilising sucrose as substrate [86]. GRAS status is desirable in an industrial setting as it becomes dramatically more expensive to set up a factory authorised to safely handle infectious agents with highly regulated containment standards. Biosafety concerns, out of necessity, are therefore a major consideration when engineering strains for superior performance. Due to naturally occurring endotoxins, wild-type E. coli strains are usually not conferred GRAS status, whereas B. subtilis is suitable for human ingestion. *Pseudomonas* sp., prime candidates for rhamnolipid production, generally demonstrate features of pathogenicity; however, some *Pseudomonas* strains, such as *Pseudomonas putida* KT2440, are designated as HV1 (FDA classification: host-vector system safety level 1), meaning it is permissible to work with this strain in a P1 or ML1 facility [87]. *Escherichia coli* K12 is similarly described as an HV1 strain and is safe to work with due to an absence of virulence factors.

While no rhamnolipid-producing organism is as yet known to be commissioned for industrial use, in 2016, Evonik Industries AG revealed that they had a

commercial rhamnolipid product in their pipeline – no mention of the strain was made, however, an existing Evonik patent [88] does list recombinant *P. putida* in its claims. Evonik is also actively marketing, through Ecover, a manufacturer of cleansers and detergents, products containing sophorolipids. The yeast, *Starmerella bombicola*, is currently the most industrially prolific strain used in sophorolipid production (volumetric productivity <3.7 g l^{-1} h^{-1} and highest titre of 477 g l^{-1}). A comprehensive mini-review of a decade of metabolic engineering of *S. bombicola* was presented in 2018 [89]. This is a useful reference for genetic modifications of *S. bombicola's* biosynthetic pathways in aid of SL efficiency improvements. A 2020 article describes the deletion, overexpression and construction of complementary mutant strains to *S. bombicola* CGMCC, specifically pertaining to the Bro1 protein. Rate-limiting enzymes in SL biosynthesis were significantly down-regulated in the *Δbro1* and the deletion mutant did not produce SLs [90].

Despite *P. putida's* reputation for being metabolically versatile and physiologically resilient in the face of exogenous pressures, under micro-oxic conditions it struggles to maintain redox balance and generate energy in the form of ATP. As an obligate aerobe, the absence of molecular oxygen hampers its potential as an industrial workhorse, which is expected to have a negative impact on biosurfactant output, considering scale-up of good oxygen transfer is difficult in bioreactors. Kampers et al. combined genome-scale metabolic modelling with comparative genomics to recombine *P. putida* strains with acetate kinase from *E. coli*, and a dehydrogenase and ribonucleotide triphosphate reductase from *Lactobacillus lactis*, to correct ATP and essential metabolite production, respectively. The team successfully used adaptive laboratory evolution to adjust computational models [91].

Chemical mutagenesis using 50 μg/10 ml ethidium bromide (EtBr) exposure for 60 min caused an *Aspergillus niger*, or black rot fungus, mutant strain (*A. niger* M2) to produce 3.3 g l^{-1} biosurfactant in comparison with the native strain output of 2.3 g l^{-1}. Oil displacement, emulsification activity and the emulsification index also increased from 49.74 to 59.81 cm^2, 1.024 to 1.262 (OD_{540}), and 57% to 62.3%, respectively. *Aspergillus niger* M2 produced a maximum of 5.50 g l^{-1} biosurfactant after optimising the solid-sate fermentation run with response surface methodology (RSM) in a central composite design (CCD) [92]. No mention of the type or classification of biosurfactants produced was mentioned in the paper.

Lei et al. used genomic fragments from *P. aeruginosa* SG to construct recombinant plasmids, which were introduced into an *E. coli* S17-1 strain by transformation, and then re-introduced into wild-type *P. aeruginosa* SG using conjugation. The team was able to identify key genes involved in rhamnolipid synthesis (*pslAB*, *phaC1DC2*) using knockout strategies targeting secondary metabolic bypass pathways (Pel, Psl and PHA). The double mutant strain, SG *ΔpslAB ΔphaC1DC2*, which was designed to generate higher levels of fatty acid and glycosyl precursors, produced 67.7% more biosurfactant than the wild-type strain, at a maximum of 21.496 g l^{-1} rhamnolipid. This could be improved by statistically optimising medium composition and fermentation parameters [93].

All this is to say that due to the variety of organisms used in biosurfactant synthesis, the number of products produced, and the relative newness of the field,

there is still significant work needed in the molecular biological optimisation of biosurfactant production.

5 Reactor Type and Operation

The type, size, shape, and metabolic requirements of the organism defines what bioreactor can be used in a process. The list of organisms producing biosurfactants is long, and the number we have identified is growing. For the most part the organisms fall within bacteria and fungi, generally single-celled and metabolising under aerobic conditions. There are of course exceptions to this, and indeed each bioprocess must be designed around a specific chassis organism, which may have particular requirements. A specific counter example to the usual aerobic production of surfactin by *B. subtilis* is that of Willenbacher et al. [94], who demonstrated anaerobic fermentative production of surfactin, with titres exceeding those achieved in aerobic fermentation. Nonetheless, most work has been done for processes fitting the description: aerobic, submerged, planktonic culture [95]; for the most part, these fermentations are done in temperature, oxygen, agitation controlled vessels – vessels specified for planktonic submerged culture [96]. The physical configuration that biosurfactant reactors take varies somewhat, with CSTRs being the most common. These various reactors can also be operated under a number of modes. Beuker et al. [97] give a good discussion on batch, fed-batch and continuous fermentation strategies.

However, normal aerated CSTRs commonly are difficult to use for the production of biosurfactants. They are:

1. comparatively expensive to operate – aeration and agitation are significant operational costs. Since they are commonly operated in batch, there is significant down-time for set-up and cleaning. Sterilisation costs before and after fermentation can be significant
2. suffer from excessive foaming [98] – ironically, the success of the fermentation produced a product which makes the fermentation difficult to perform. Excessive foaming often overflows CSTR reactors, despite significant use of antifoam and foam-breakers
3. at high cell concentrations can become oxygen limited – the volumetric productivity of a reactor can be improved at high cell concentrations, but since biosurfactant production requires oxygenic metabolism, if the concentration of metabolising cells in the reactor gets too high, areas of oxygen depletion can occur
4. run into a number of complications when scaling up – specifically with regard to mixing and sparging: as one scales up, volume increases cubically, while required power (for mixing or sparging) increases by power 5 [99].

In order to circumvent some of these issues, and to improve productivity some modified reactor geometries specifically for biosurfactant production have been proposed in the literature:

5.1 Foam Fractionation Reactor

Conventional cultivation techniques for biosurfactant production often suffer from severe foaming, which can even crash fermentations. To combat this foam breaking agents are commonly employed, such as antifoam chemicals added to the fermentation broth, or mechanical foam-breakers in the headspace. These interventions add complexity and cost to the process and can reduce productivity (particularly in the case of chemical antifoam agents which can interfere with the organisms' metabolism). An alternative approach is to use the foam formation rather than try to prevent it. Biosurfactants (which cause the foaming) collect at gas–liquid interfaces, and so are concentrated in the foam. The foam can therefore be used as a separation tool for in situ product removal during fermentation.

Reactors have been designed and demonstrated which utilise this principle; called foam fractionation reactors [100], they include a tube above the fermentation vessel, and a foamate collection vessel. The riser tube is designed such that the foam, containing the biosurfactant (and a significant amount of culture medium) emerges as a column. Bubble coalescence and foam stabilisation occur through this riser tube, while the entrained culture medium trickles back down into the reactor vessel. The foam, which is now concentrated in surface active compounds, then moves to the foamate collection vessel, where a foam-breaker is employed to destabilise the foam back to a liquid phase. Often either a mechanical foam-breaker is used or low pressure is used to burst the foam. The process separates compounds with low surface activity from high, such that the collected foamate is rich in biosurfactants, and can be sent for further processing: indeed, processing can even occur in the foamate collection vessel: Anic et al. [101] demonstrated the integration of an adsorption column in the foamate collection vessel, so that foamate (now poor in product) could be recycled back to the fermentation vessel. Najmi et al. [102] have a good discussion on foam fractionation and the parameters affecting biosurfactant collection using it.

Foam fractionation has been demonstrated in a number of biosurfactant production studies. Chen et al. [103] used foam fractionation to concentrate surfactin from *Bacillus* cultivation 50-fold over the culture medium, while Perna et al. [104] developed models to describe surfactin collection using foam fractionation and showed 30-fold concentration. Both Khondee et al. [105] and Yi et al. [106] combined foam fractionation with cell immobilisation to improve surfactin productivity, although the volumetric productivity is still far short of that seen in rhamnolipids and sophorolipids.

Rhamnolipids have also been demonstrated to be concentrated via foam fractionation, although the organisms used to produce these compounds tend to collect in the

foam, reducing productivity. Anic et al. [101] integrated foam fractionation and adsorption of rhamnolipids, which improved productivity by allowing cell recycling, and resulted in a claimed 100% rhamnolipid recovery. Zheng et al. [107] linked cyclic fermentation to foam fractionation, with a 75% recovery of produced rhamnolipids while Long et al. [108] demonstrated an 83% increase in productivity when using foam fractionation (fitted with a valve foam-breaker) to recover the rhamnolipids. Bueker et al. [109] demonstrated excellent rhamnolipid recoveries, with low biomass removal, potentially due to the heterologous host used – *P. putida* instead of *P. aeruginosa,* which appeared to have a lower affinity for bubbles. Xu et al. [110] demonstrated the use of a novel foam-breaker methodology, to improve rhamnolipid productivity, which although they did not use the apparatus for foam fractionation, could be implemented in that fashion.

Foam fractionation of sophorolipids also suffers from significant cell entrainment in the bubble phase, however, there has been some work using the configuration for in situ product recovery, when cell recycling can be enacted. Liu et al. [111] used a modified version of foam fractionation where they added a second oil phase to collect the sophorolipids, before using foaming to collect the sophorolipid-rich oil and separate it from the culture medium, which they recycled. This methodology aligns well with other in situ solvent extraction based systems, such as those implemented for acetone-ethanol-butanol fermentation [112].

Trehalolipids separation using foam fractionation has been demonstrated, with Bages-Estopa et al. [113] using a hexadecane substrate which suppresses foaming during fermentation. When the substrate is exhausted, the trehalolipid product caused foaming, collecting the product in the foamate, cleverly combining substrate exhaustion with product recovery.

A significant limitation of this methodology is that many media components in biosurfactant fermentation themselves have a hydrophobic activity, and so also partition to the foamate, removing them from the reaction volume. Further, the organisms themselves often also become entrained, reducing biomass concentration. Additionally, because the productivity of biosurfactants changes through the course of the fermentation, the amount of liquid lost to foam fractionation changes over time. This poses control issues for a bioprocess, as highlighted in Chenikher et al. [114]. These issues can be overcome if the foamate can be depleted in product and then recycled, or if the cells can be held within the reactor and prevented from partitioning to the gas–liquid interface. This can be achieved through immobilisation or through a biofilm. But where foam fractionation really becomes a viable process option is when the product is preferentially partitioned to the foam phase, while cells and media components are not. This is very much a function of solution properties (such as pH and salinity), and the chassis organism used. With the advent of powerful molecular biological tools, suitably hydrophilic organisms might be engineered to produce the desired products and linked to foam fractionation reactors. Nonetheless, the methodology has significant merit – combining fermentation with the first step in downstream processing.

5.2 Membrane-Based Reactors

There are two main configurations where membranes can become integral parts of a biosurfactant fermentation [115]: (1) the membrane is used to retain active biomass within the reactor, while product-rich or substrate-depleted medium is removed through the membrane, or (2) the membrane is used for oxygenation of the reaction vessel. The second configuration has been more commonly demonstrated in biosurfactant production studies than the first – with little work specifically demonstrating the retention of biosurfactant-producing cells through the use of a membrane. However, this is not necessarily an indication that membrane cell retention is not a viable process option: indeed, there is an implicit bias in laboratory-based experimentation towards batch processes (they are easier to conduct at laboratory scale), while a membrane reactor for cell retention is more favourable for continuous production studies.

A major process operational consideration in biosurfactant production, as has come up several times in this discussion, is that of excessive foaming when a sparged reactor is used. The utilisation of a membrane for oxygenation is one potential solution to this difficulty: oxygen or air can be passed through solid membranes within the fermentation medium, allowing oxygen to diffuse into the liquid (or emerge as small bubbles). However, to get equivalent oxygen transfer from membranes rather than using sparging requires significant membrane area, or oxygen partial pressure. Additionally, membranes are comparatively expensive, relatively delicate, and liable to becoming blocked by organisms growing on the membrane surface.

Coutte et al. [116] demonstrated the use of oxygenation using hollow fibre membranes for the production of surfactin and fengycin by *Bacillus*, with some success, and then went further to demonstrate continuous surfactin production [117], using both membrane oxygenation and membrane-based cell recycling. Although a they did note the tendency for the surfactin to adsorb onto the membrane, reducing oxygen transfer rates. Motto dos Santos et al. [118] built on this work, using the same reactors, to examine media limitations on surfactin production and to develop a high-throughput methodology for media testing. Noting the issue of substantial surfactin adsorption onto the oxygenation membranes, Behary et al. [119] modified PET membranes to reduce adsorption, with some success. Beth et al. [120] took a more fundamental approach, through modelling the gas–liquid mass transfer which occurs in these reactors. Pinzon et al. [121] demonstrated the production of rhamnolipids using a membrane-based approach, but utilising nitrate as an oxygen source rather than molecular oxygen.

These types of reactors show good promise, but comparatively little work has been done in the field: there is clearly still significant work to be done in this field – both in terms of membrane-based oxygenation and membrane-based cell retention.

5.3 Slurry Bioreactors

Slurry reactors have for the most part been used in the treatment of petrochemical contaminated soils [122]. The aim of these processes is not to produce a biosurfactant product, but rather to use biosurfactant-producing organisms in the bioremediation of contaminated soil [5]. As such, these reactors operate with a slurry of soil, culture medium, aeration and (often inoculated) microorganisms. Robles et al. [122] present a good review of the application of slurry reactors for the treatment of recalcitrant contaminants in soil, which often involves the use of organisms which produce biosurfactants. While this work is useful and important, it is unlikely to give rise to an economical method for large-scale purified biosurfactant product.

5.4 Solid State Fermentation

Solid state fermentation is based on the use of a solid substrate phase (commonly an agri-waste [123] or other low-value substrate [124]), which is inoculated with the desired organism and kept at growth conditions. After a period of fermentation, in which the organism proliferates, consumes those parts of the substrate which are degradable (often a liquid substrate is also added, should the solid material be low in fermentable compounds) and produces the desired product. In the most commonly used procedure a batch fermentation is followed by extraction of the product from the solid via solubilisation in a selective solvent, ending the fermentation. There is some disagreement in the literature on the exact definition of 'solid state fermentation': some consider SSF to only include solid substrates which are themselves at least partially fermentable (substrates such as agro-processing wastes [125]), while others consider fermentations conducted using liquid substrate, but inert solid media (such as polymer solids) to also be considered. The distinction is semantic, but the choices between solid support do have cost and processing implications; for instance, choosing agricultural wastes as a solid support can be cost-effective, but can also introduce impurities and complicating compounds to the product solution, which then will require further downstream processing.

Solid state fermentation avoids the issue of excessive foaming, since minimal liquid is present, and oxygenation can be easily achieved by passing air over the substrate solid. The technology also limits operational expenses such as agitation, liquid composition control and sparging associated with submerged culture [124].

Rhamnolipid production via solid state fermentation has been demonstrated by Camilios-Neto et al. [126], and El-Housseiny et al. [127] using agro-processing wastes, which produced comparable or improved concentrations of rhamnolipid product, when considered on a per volume of liquid basis, but which underperformed standard submerged cultures significantly on a per volume of reactor basis. Gong et al. [128] utilised a polyurethane solid support on which to

grow *Pseudomonas* on a palm-oil substrate, to produce 39.8 g/l of rhamnolipid product. Lopes et al. [129] demonstrated co-culture of ethanol and rhamnolipid-producing organisms on sugarcane bagasse, producing 9.1 g/l of biosurfactant product. Co-culture has several economic and process design implications that have yet to be fully explored in the literature, but which provide interesting potential for a biorefinery-type approach to biosurfactant production.

Sophorolipid production using SSF was demonstrated by Jiménez-Peñalver et al. [130], first using an inert polymer foam support and molasses and stearic acid as substrate, and then on sunflower oil agro-processing waste [131]. They achieved a fairly good yield, and reasonable congener distribution, but the product titre was low in comparison with submerged culture production of sophorolipids, which can reach extremely high levels [132].

Slivinsky et al. [133] produced surfactin via SSF on sugarcane bagasse-okara support, producing 0.8 g/l of surfactin per volume of impregnating liquid, while Kumar et al. [134] demonstrated iturin A production of 0.8 g/l using SSF based on sunflower oil cake, another agro-processing waste material. While these productivities are comparatively high when considered on the liquid volume basis, they are low when considering the reactor volume as a basis. Lourenço et al. [135] utilised *Trametes versicolor*, an organism not commonly reported to produce biosurfactants, to produce an unidentified lipid- and protein-containing biosurfactant on olive mill solid waste using SSF.

Faria et al. [136] demonstrated the production of MELs using SSF under a variety of conditions (with or without pre-treatment and simultaneous saccharification) giving productivities of 4.5 g/l, far below the best submerged culture productivities (which can exceed 100 g/l: see Beck et al. [137] for an excellent review of MELs).

Other biosurfactants have also been reported to be produced using SSF. Velioglu and Urek [138, 139] cultivated *Pleurotus djamor* on a variety of solid substrates, to produce a water solubilised extract containing an unknown biosurfactant. While Brumano et al. [140] reported production of an unidentified biosurfactant (potentially glycerol-liamocin, considering the use of *Aureobasidium pullulans* [141]) on bagasse-based SSF. Rubio-Ribeaux et al. [142] demonstrated the production of an unidentified biosurfactant from *Candida Tropicalis* cultivated on food waste and crude glycerol, however, due to the unidentified compound, no comparisons to other processing methods can be drawn.

In all, SSF is a processing route with significant potential, particularly in the use of waste agricultural residues, and in the use of filamentous fungi. However, significant strides in process economics, scale-up, and increased product titre will likely be needed for this to become a viable industrial process route.

5.5 *Immobilised Cell or Biofilm Based Reactors*

There are a number of potential benefits to utilising a bioreactor based on immobilised cells, or cells adhered to a solid support, usually in the form of a

biofilm. The methodology de-couples hydraulic retention time (the period of time the medium spends in the reactor) from solids retention time (the period of time cells and other solids spend in the reactor). This can allow the development of either fully continuous, or fill-and-draw operational strategies.

Further, the biology of the fermentative organisms often shifts significantly when growing under biofilm-forming conditions, rather than planktonically. This can result in a shift in product distribution (as was shown by the increased production of fengycin in the work of Chtioui et al. [143]) or increased yield.

Depending on the physical configuration of the reactor in question, immobilisation/biofilm can also be a strategy to prevent foaming, by reducing the need for sparged oxygen. Solid supports can allow more direct contacting of the gas phase within the reactor, such as in a drip-flow reactor, however, it also decreases the effective productive volume as a smaller portion of the reactor contains productive cells.

Additionally, the continued operation of continuous fermentation requires stationary phase production of the desired products – this is not always the case, and so organism and strain selection as well as media composition can have a large role here. For instance, Sodagari and Ju [144] demonstrated a restart in stationary phase rhamnolipid production through replenishing nitrogen sources in the media; although this was only required periodically and not continuously. Investigations into the implications of continuous production are sorely needed in the literature to bolster our understanding of stationary phase metabolism and reactor operation.

There are a number of physical configurations in which researchers have demonstrated the applicability of immobilised or biofilm based cells for biosurfactant production:

5.5.1 Immobilised Cells

It is the case that several biosurfactant-producing organisms do not form biofilms, and so an alternative strategy is needed for biomass retention. One route, which is commonly used in biotechnology, is the entrapment of cells within a solid matrix. This matrix, usually in the form of millimetre-scale beads, can then be retained within the reactor, allowing flow-through of medium. Comparatively high biomass loading can be induced, through cell loading in the beads, although cognisance must be taken of potential mass transfer limitations of substrates into the beads and product out: concentration gradients can induce metabolic variation through the bead cross-section.

Khondee et al. [105] have demonstrated the immobilisation of *Bacillus* in chitosan-based hydrogel beads, followed by product recovery with foam fractionation. They showed how these beads could be recycled for several fermentations with stable surfactin production. Ohadi et al. [145] encapsulated *Bacillus licheniformis* in alginate, for the production of unidentified biosurfactants.

In a different processing route choice, Hidayat et al. [146] immobilised active enzymes on Amberlite resin beads, for chemical production of fructose oleic ester

biosurfactants. The idea is an attractive one: instead of immobilising the whole organism, can the enzyme operate alone? This depends on the enzyme in question, and unfortunately most biosurfactants require a cascade of enzymes not a single one.

Kebbouche-Gana et al. [147] investigated the immobilisation of a halophilic *Natrialba sp.* in alginate beads, to produce an unidentified biosurfactant. As has been discussed in the section on strain selection, there are a number of organisms identified which produce biosurfactants, and a large part of producing an economically viable process is choosing an appropriate chassis organism. These authors' use of a halotolerant organism speaks to the possibility of using extreme culture conditions, to prevent contamination.

Abouseoud et al. [148] followed by Onwosi and Odibo [149], following a similar procedure, demonstrated immobilisation of *Pseudomonas* in alginate gel beads, for rhamnolipid production. Abouseoud [148] clearly illustrated one of the limitations in immobilised cell bioprocesses – that of diffusion limitations. In the case of biosurfactants in particular, the substrates required to diffuse through the matrices are alkanes or fatty acids. These compounds can exhibit slow diffusion characteristics, and thereby limit productivity. On the positive side, immobilised cells can be recycled: Jeong et al. [150] showed rhamnolipid production, through 15 cycles with well-maintained productivity, of *Pseudomonas* immobilised in polyvinyl alcohol hydrogel. This material has very good mechanical properties for cell immobilisation in a number of fields [151], in contrast to the more widely used alginate gels, which are comparatively delicate and susceptible to chemical dissolution. Work by Heyd et al. [152] focussed on the cycling of immobilised cells, through the integration of magnetic separation for magnetic hydrogel beads containing *Pseudomonas* for recycling of biomass in a process intensification strategy.

5.6 *Biofilm Support*

Brück et al. [153] used genetically modified *Bacillus* to improve biofilm adhesion, which then resulted in improved lipopeptide productivity when using laboratory scale drip-flow reactors. Vandermies et al. [154] similarly demonstrated variable cell adhesion after genetic manipulation of *Yarrowia*. Adhesive cell strategies would allow for continuous flow-through of medium and collection of product, as demonstrated in this drip flow reactor: the method has potential for reduced operational cost at scale.

Brück et al. [155] demonstrated the production of lipopeptides using a trickle-bed bioreactor, filled with structured metal packing on which Bacillus developed a biofilm, while Vandermies et al. [154] similarly demonstrated biofilm establishment of the yeast *Yarrowia* on comparable structured packing. Zune [156] demonstrated the production of surfactin (via *Bacillus* [157]), hydrophobin (via *Trichoderma* [158]) and recombinant proteins (via *Aspergillus*) using a trickle-bed structured packing bioreactor system. The trickle-bed configuration demonstrates excellent

oxygen transfer, while avoiding excessive foaming, and has the potential to be developed into a continuous process.

Fahim et al. [159] allowed polymer beads to be colonised with *Bacillus* biofilm, before insertion of the beads into an inverse fluidised bed reactor. This configuration allows for excellent oxygen transfer, but may also succumb to significant foaming.

5.7 Rotating Disc Bioreactors

While technically a specific configuration of a biofilm reactor, they differ in their moving components and air–liquid contacting from most other configurations. In this configuration, solid supports, in the form of rotating disks, on which biomass grows in a biofilm, rotate between growth media and the gas phase. Generally growth of cells in the liquid medium is quickly limited by oxygen transfer, and so the majority of biomass grows adhered to the disks. This configuration prevents excessive foaming, and the biosurfactant product can accumulate in the liquid media, which is useful if a continuous or semi-continuous process is to be used. However, these reactors suffer from a number of limitations: biomass is limited by disk area, effectively reducing productivity. Further, only organisms which form biofilms can be used in these systems (unless an additional immobilisation step is employed); several of the biosurfactant-producing organisms do not form biofilms, and so without genetic modification (as was done by Brück et al. [153]), these organisms could not be used with this technology.

Few researchers have demonstrated the use of rotating disk bioreactors for the production of biosurfactants: Chtioui et al. [143] demonstrated production of surfactin and fengycin lipopeptides, with their later work [160] improving fengycin concentrations achieved up to 0.8 g/l fengycin concentration and 88% selectivity (the remainder being surfactin), a comparatively high concentration for fengycin. Amin et al. [161] used the vertical rotating immobilised cell reactor, developed by that research group in the 1980s [162], to immobilise *Bacillus* on polyurethane foam support, for surfactin production used in a linked bio-desulphurisation process. No reports of other biosurfactants made using this or similar technology are available, which may either speak to the limitations of the technique or its relative novelty.

5.8 In Situ Extraction Reactors

Dolman et al. [132, 163] have developed a reactor which utilises a liquid–liquid phase separation between a sophorolipid-rich phase and a cell-rich phase. This reactor includes an in situ settler volume for gravity settling of the two phases, allowing continuous collection of product and recycling of fermentation medium [163]. Similarly, Wang et al. [164] have built on the idea of self-separating sophorolipid-rich phase, based on *S. bombicola* cultivated on food waste as a

substrate. Zhang et al. [165] developed a methodology for product separation, as well as cell and substrate recycle.

This work highlights a difficulty in implementing in situ extractive systems with biosurfactant production: most of these fermentations include an immiscible oil phase as a substrate, and so a solvent extraction system (the most widely implemented in situ extraction methodology) would not be suitable for biosurfactant fermentation.

Nonetheless, the potential for in situ product recovery is an attractive one for the operating engineer – it allows continuous processing, and a consistent (and preferably high) rate, prevents product inhibition or post-fermentation consumption of product. In all, in situ reactors provide an excellent opportunity, which should be further pursued.

6 Media Composition

Much work has focussed on the use of different carbon sources for cultivation of biosurfactant production, and there are several excellent reviews which focus on this aspect of biosurfactant productivity to which the reader can refer for a more in-depth analysis than that presented here [81, 166–175].

The work on media development has been driven primarily by two forces: firstly, the carbon source makes up a significant portion of the cost of cultivation, and so the logic is that using cheaper substrates will improve the economics. This is certainly true, and several authors have shown fairly good productivities on such substrates as waste materials, agro-processing by-products and other low-value substrates. Table 3 gives a non-exhaustive list of some of the substrates used for the production of biosurfactants. Substrate cost can make up a significant portion of the total cost, particularly when pure or virgin substrates are used. Indeed, it is not quite true that the cheapest substrate is free – through integrating valuable compound production with the treatment of a waste stream or wastewater, the cost of wastewater treatment can be offset by the product sale. Giving rise to an integrated wastewater biorefinery [176].

However, the use of waste materials is not without limitations – a significant area of concern is consistency: waste materials can vastly vary in composition, which can give rise to variable product compositions. Further, contaminating components from the waste substrates may require further downstream processing steps to remove – these additional steps could be as, if not more, costly than the savings from using the waste substrate in the first place. And finally, depending on the final use of the biosurfactant, there may be market resistance to using waste as substrate. For instance, MELs are predominantly used in cosmetics [70, 177], a high value market that may object if the MELs were produced from, for example, food waste [178].

The second drive in the choice of substrate is the desire to limit congener variability within a batch. By limiting the feedstock to a single fatty acid [224], for instance, the biosurfactant congeners can be limited to incorporate that fatty acid

Table 3 Summary of some key low-value substrates used for biosurfactant production

Substrate		Biosurfactant produced	Reference (s)
Lignocellulosic biomass	Lignocellulose	Rhamnolipids, sophorolipids, trehalolipids, MELs	[179–184]
	Hydrolysed wood or paper	Rhamnolipids, lipopeptide	[184, 185]
Food waste	Kitchen waste	Lipopeptides, biosurfactant, sophorolipids	[79, 182, 186–188]
	Molasses	Biosurfactants	[189]
	Potato waste	Lipopeptides, rhamnolipid	[123, 190, 191]
	Fish residue	Lipopeptide	[192]
	Grapeseed flour	Lipopeptide	[193]
	Brewery waste	Lipopeptide	[194, 195]
	Whey	Biosurfactant	[196]
	Cassava wastewater	MELs	[197]
	Coconut water	MELs	[198]
	Pineapple waste	Lipopeptides	[199]
Vegetable oils	Palm-oil mill effluent	Rhamnolipids	[200–202]
	Cooking oil	Rhamnolipids, MELs, glycolipids, biosurfactants, lipopeptide, sophorolipids	[178, 203–215]
	Biodiesel waste	Biosurfactants	[216]
	Olive mill waste	Rhamnolipids, lipopeptides, biosurfactant	[135, 217, 218]
	Coconut oil cake	Rhamnolipids	[219]
	Sunflower oil cake	Sophorolipids	[131]
	Glycerol	Sophorolipids, rhamnolipids	[220–223]

moiety [215], or a greater degree of control over congener distribution can be achieved [225, 226]. This does not entirely limit congener production, since the organism is still able to produce its own fatty acids (of varying lengths), and interconvert between those is metabolises, but the strategy may improve product composition, and inter-batch variability. This is a comparatively unexplored area of biosurfactant metabolism, which could improve the cost of bringing a product to market through reducing downstream processing costs, and producing a more consistent and higher purity product.

Beyond these two major considerations in media optimisation, there are other factors that should be considered with regard to media composition. While a carbon source may be supplied from a low-value waste substrate, other macro-nutrients also required by the organisms: nitrogen and oxygen predominantly. Indeed several

researchers have noted that the producing organisms' metabolism may be driven towards increased biosurfactant production if the C:N ratio is tuned correctly [227–230]. And a major process control constraint is dissolved oxygen concentration – affected by aeration rate and agitation.

Micronutrients are also important for optimal biosurfactant productivity. The enzymes which catalyse the metabolism of these products are metal-containing (manganese [231], zinc and iron in particular, but also including a host of other metallic and other compounds [232]), and so at least sufficient amounts of those micronutrients are needed. However, the question does not appear to be simply one of stoichiometry, with a more complicated physiological response to metallic micronutrients in the organism [233, 234].

The media environment can have an inordinate and difficult to predict effect on biosurfactant productivity – Wang et al. [235] even demonstrated that simply using tap water instead of distilled can change sophorolipid productivity. This sensitivity is extremely difficult for the bioprocess engineer operating a fermentation: control of sensitive systems is paramount, and if the system is unstable, control is virtually impossible.

In general there has been a significant focus on media optimisation in the literature – there are several examples of multifactorial investigations to find the 'optimum' concentration of various components in biosurfactant production. This is useful work, but a significant limitation is that optimal media compositions are extremely species (and even strain) dependent. And so, media optimisation should be one of the last factors to be considered in the development of a process.

7 Downstream Processing

Downstream processing often accounts for a significant (if not the majority, if not the vast majority) of a bioprocess's cost. The level of purity required for a product defines the required levels of purification needed, and in general a process will only purify to just within specification and no more. And of course, the purification method must not damage the activity of the biosurfactant – there is no point in purifying a product which no longer displays the desired activity. Thus product stability through the process is a significant consideration in downstream processing; biosurfactants are comparatively stable, so many purification routes are viable. Many studies, however, do not verify activity post purification – this is a significant gap that needs further validation by researchers in separations.

During purification, a series of unit operations are commonly employed, to remove contaminating compounds via each potential difference from the desired product. And so any discussion of purification will not simply consider a single unit operation (unless you are lucky enough to find a single operation which selectively separates *only* the compound of interest – an unlikely occurrence). Commonly separation proceeds from easiest to separate, to most difficult, and in the case of biosurfactants the most difficult to separate is commonly congeners of the same

Table 4 Separation methodologies employed for biosurfactant purification

Method	Mechanism of separation	Reference(s)
Gravity settling	Density	[132, 163]
Adsorption	Adsorption	[237–239]
Precipitation (acid, salting out or solvent)	Solubility (solid–liquid)	[240–244]
Solvent extraction	Solubility (liquid–liquid	[245–248]
Filtration	Size (of molecule, crystal and micelle)	[102, 197, 249, 250]
Foam fractionation	Hydrophobicity	[102]
Chromatography (column, TLC, gas)	Diffusion speed through medium (based on size, charge, hydrophobicity, binding, ligation etc)	[251–253]

biosurfactant. If congener purity is not essential, an impure product is cheaper to produce.

This discussion will not aim to examine all the purification methodologies employed by researchers in biosurfactant production, both since most processing options are standard purification technologies which can be found in most bioprocessing textbooks. And further, many laboratory-based studies utilise purification routes which are unlikely to be feasible at large scale. Indeed, there are a few reviews which deal with this topic specifically, to which the reader might refer. Clarke and Ranganjaran [236] give a good review of some of the more common purification routes used, specifically for lipopeptides.

Nonetheless, a summary of some purification methodologies that have been demonstrated, and the basis for separation, may guide the reader's thinking with regard to major unit operation choices. Table 4 summarises processes demonstrated on biosurfactants, with detail on the principle behind the separation. Of course, in many of these studies several purification methods were employed, in series, in order to achieve the required purity. Some of the methods tabulated here might be scaled to industrial levels, but many are only really useful at laboratory scale:

8 Final Use and Market

The deployment of a product to market relies not only on sound science and engineering, in developing and enacting a production process, but also on a number of following steps: how is the product packaged and stored? Is it shelf-stable for long? Does it require formulation with stabilisers or binders? If it is produced as powder, can it be spray dried, lyophilised or must it be dried under low impact conditions.

These are the sorts of questions which are not commonly answered in the academic literature, and fall more within the wheelhouse of production companies. However, these downstream choices can have implications for upstream process

choices, and so should be considered as soon as is reasonable. Nonetheless, there is some work in the literature to which one can refer:

Freitas et al. [254] demonstrated the production of a biosurfactant by *Candida bombicola*, and the subsequent formulation of a product with inclusion of potassium sorbate as a stabiliser, and using tyndallisation as a sterilisation methodology. The product retained its biosurfactant properties even after 120 days of storage. Soares Da Silva et al. [255] used a similar methodology to Freitas on biosurfactants produced by *Pseudomonas cepacia*, with biosurfactant activity retained. Almeida et al. [256] produced biosurfactant using *Candida tropicalis* formulating a product with potassium sorbate as preservative. They investigated product stability over 120 days under varying conditions of pH, temperature and salinity and found the product to retain its biosurfactant properties. Salek and Euston [67] have an excellent review on bioemulsifiers, including their stability, as a particular application of biosurfactants.

One of the major opportunities of biosurfactants is the variety of functional groups which they can include. The same family of biosurfactants can have hundreds of congeners, which may have quite different physical properties. These properties have hardly been explored, with much literature and industrial work done on a comparatively low number of congeners. This is hardly surprising – the science here is complex, getting pure congeners is exceedingly difficult, and the relative youth of the industry means that low hanging fruit have been sought first. But this is an area where much work might yet be done – producing congeners with varying composition, and examining their activities. But to achieve this the fields of strain selection, modification, media control and certainly congener separation have much work to do. However, once new compositions and properties are investigated, invariably new markets and uses will be found which fit with these properties.

9 Conclusions

This article has highlighted some key considerations when developing a bioprocess based on microbial production of biosurfactants. The state of the art for many of these considerations is critically presented, with consideration for specific gaps in the literature, and areas which may benefit from further work. It is clear that the field of biosurfactant research is growing, commensurately with the growing market and demand for these products. Further, the structural diversity of these compounds (even within a particular class) lends itself to new application discovery. The significant limitation of the field at the current time is the relative expense of (1) production of the compounds (considering substrate costs, reactor operation, and product yield and titre) and (2) downstream processing costs. Nonetheless, many researchers around the world are investigating aspects of this field, and significant advances are made continuously.

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Environmental Impacts of Biosurfactants from a Life Cycle Perspective: A Systematic Literature Review

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Contents

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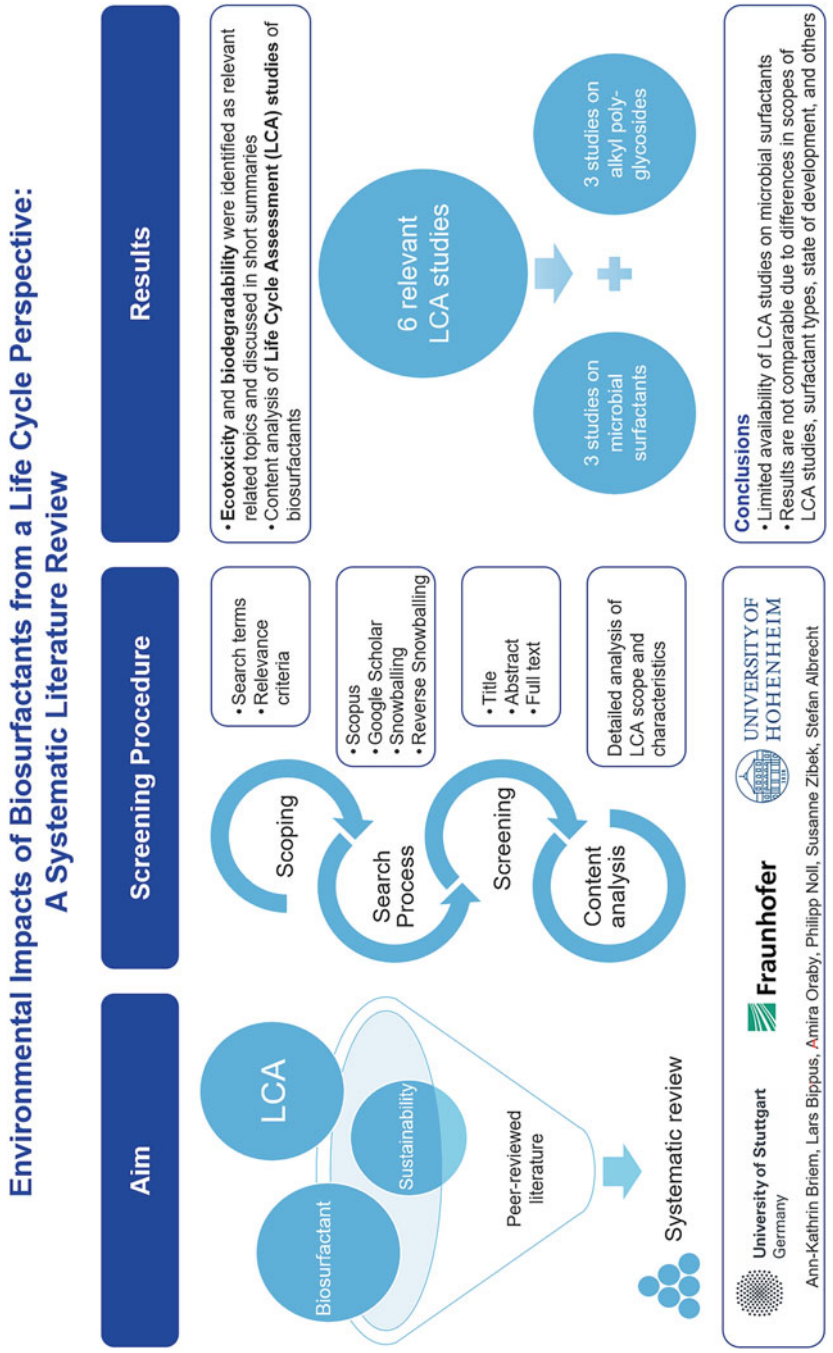
Abstract Biosurfactants are considered as an environmentally friendly and sustainable alternative to conventional fossil-derived and chemically produced surfactants. Their production pathways, physicochemical properties, and applications are widely researched and discussed in literature. In this context, investigating the different impacts from the entire life cycle of biosurfactants is important to understand and mitigate potential environmental hotspots. Life Cycle Assessment (LCA) is an internationally accepted and standardized methodology to analyze the environmental impacts of products from a holistic view. Therefore, this study provides a detailed overview of existing LCA studies of biosurfactants by means of a systematic literature research. The focus specifically lies on articles that investigated microbial biosurfactants. However, the systematic approach used ensured a broader overview related to bio-based surfactants as well. Furthermore, two related topics, ecotoxicity and biodegradability of biosurfactants, were identified and discussed based on the search findings. After screening over 2,500 documents using Scopus and Google Scholar, six relevant LCA articles of biosurfactants could be identified. The identified articles are divided into LCA studies of alkyl polyglycosides, chemically produced bio-based surfactants, and LCA studies of microbial biosurfactants, their content analyzed and discussed in context. In conclusion, the number of available LCA studies is very limited and their results are often not comparable. To the best of the authors' knowledge, this review is the first of its kind to provide a detailed overview of LCA studies of biosurfactants. Consequently, the need for implementing more LCA studies becomes clear.

Graphical Abstract



Keywords Alkyl polyglycoside, Bio-based, Biodegradability, Bioeconomy, Biosurfactant, Ecotoxicity, Environmentally friendly, LCA, Life cycle assessment, Microbial surfactant, Renewable resources, Rhamnolipid, Screening, Sophorolipid, Sustainability, Systematic literature research

1 Introduction

The Paris Agreement and the United Nations Sustainable Development Goals are two recent examples of global efforts to work together towards a sustainable future [1, 2]. Furthermore, the combat against climate change and the implementation of sustainable solutions regarding social, environmental, and economic aspects have become an urgent international mission, for example, reflected by the European Green Deal [3]. In doing so, the European Union follows various strategies, such as the "Bioeconomy Strategy" [4]. The concept of bio-economy "encompasses production of renewable biological resources and their conversion into food, feed, bio-based products and bioenergy" [4]. It addresses numerous industries, such as the chemical and biotechnological industry, where solutions are being investigated, demonstrated, and scaled up from laboratory to industrial scale [4, 7, 8]. Biosurfactants are one example of a bio-economic product. While chemically produced surfactants can also be bio-based, the term biosurfactant usually refers to microbially produced surfactants [5, 6]. Various types of biosurfactants can be produced utilizing the metabolic processes of microorganisms, such as bacteria or fungi. Depending on the molecular weight of microbial amphiphilic metabolites, a distinction is made between high-molecular-weight amphiphilic polymers or bioemulsifiers, such as polysaccharides, and low-molecular-weight biosurfactants, such as glycolipids and lipopeptides [9]. Widely studied glycolipids include rhamnolipids (e.g., by *Pseudomonas aeruginosa*) [9], sophorolipids (e.g., by *Candida bombicola*) [10], as well as mannosylerythritol lipids and cellobiose lipids by various *Ustilaginaceae* species [11, 12]. Lipopeptides include surfactin and fengycin produced by *Bacillus subtilis*, among others [13]. The applications of biosurfactants are as diverse as their chemical structures and range from laundry detergents, household cleaners, cosmetics, and pharmaceuticals to bioremediation [14–21]. Some biosurfactants are produced on industrial scale and are commercially available, such as sophorolipids and rhamnolipids [6]. More biosurfactants showing promising properties and potentials for a sustainable production and respectively a wide range of applications are studied and lab-scale processes are developed, such as mannosylerythritol lipids, cellobiose lipids, surfactin, and polymyxin [5, 7, 16, 22, 23]. An excellent overview over bio-based surfactants is given in the book by Hayes et al. [20].

When the potential benefits of biosurfactants regarding environmental sustainability are pointed out, they are often referred to as being biodegradable, non-toxic, generally eco-friendly, and considered having a low overall environmental impact

[5, 6]. These attributes are often associated with bio-based products, however, not automatically true in every case. For example regarding toxicity, it can be mentioned that biosurfactants often show a low(er) toxicity compared to chemical surfactants [24–29], but referring to them as non-toxic is incorrect. Separate and more detailed investigations of the specific properties of each biosurfactant are needed to allow these statements and cannot generally be applied to all biosurfactants. Furthermore, the above-mentioned terms refer to different levels of the product; some describe inherent properties of the product, such as biodegradability, others refer to the production process or overall life cycle of the product, such as "having a low environmental impact" [6, 30].

Taking the entire life cycle of a product into account is a common approach to identify process steps within its production and disposal/use phase with high environmental impacts and derive recommendations for effective measures to improve the sustainability of products. In order to do this, Life Cycle Assessment (LCA) is an internationally established and standardized method with life cycle thinking at its core [31, 32]. LCA can quantify the environmental impacts of a product or service in regard to various environmental issues, such as climate change, acidification and eutrophication, etc. The obtained results can be used for holistic assessments and to avoid a shift of burdens from one life cycle phase to another. Consequently, researchers and companies alike can learn how to improve their production processes and how to use a product more sustainably. In the case of chemicals, LCA studies are often conducted using a cradle-to-gate approach focusing on their production, as there are often many different possible applications strongly influencing the use phase [33]. Accordingly, a recent study investigated and presented a Life Cycle Inventory dataset that represents a European average of the production of conventional surfactants and raw materials (including coconut and palm oil) for the reference year 2011 [34]. The dataset was developed in cooperation with 14 companies emphasizing the relevance of LCA studies for industrial stakeholders. However, the relevance of the application and, consequently, the influence of the use phase on the overall environmental impacts should not be neglected when following the life cycle approach.

This study aims at providing a detailed overview of the existing LCA studies of biosurfactants by means of a systematic literature research. The focus specifically lies on studies that investigated microbially produced surfactants. However, the systematic approach used ensures a broader overview related to bio-based surfactants as well, where renewable resources are converted with chemical methods. Additionally, the fields of ecotoxicity and biodegradability of biosurfactants are summarized and discussed in relation to their relevance from the life cycle perspective. Relevant LCA studies related to the production, use and disposal of biosurfactants are identified and put into context with the life cycle perspective. The studies are compared on the basis of a content analysis. Research gaps and requirements for future research are identified. In summary, this article aims to provide a thorough literature review of existing LCA studies of microbially produced surfactants and bio-based surfactants. Furthermore, recent findings are pointed out and future research perspectives are identified.

2 Systematic Literature Research Approach

In order to provide a complete overview of existing LCA studies, a detailed and systematic approach was followed during the research for this study. First, relevant search terms were defined and combined. Second, relevance criteria were defined to identify the relevant documents. Third, a predefined screening procedure was followed. Finally, the identified studies' content was analyzed regarding predefined characteristics. This entire process is described in detail in the following paragraphs.

2.1 Search String Combinations

The literature research was carried out using the scientific search engines Scopus (scopus.com [35]) and Google Scholar (scholar.google.com [36]) and was performed in January and February 2020. The search strings used for the research consisted of combinations of two or three search string components. The components are associated with biosurfactants and sustainability assessment studies. Although "biosurfactant" seems to be the most commonly used term, there are different notations and expressions for referring to microbial biosurfactants. Therefore, a number of terms for microbial biosurfactants and different spellings were used for the search. The same applies to the terminology surrounding LCA. Numerous variations were used as search string components, for example "TITLE-ABS-KEY ("microbial surfactant" AND "life cycle assessment")." All components used for the search string combinations are shown in Fig. 1. 70 different search strings resulted from combining the previously defined components. The results of these 70 document searches were then screened.

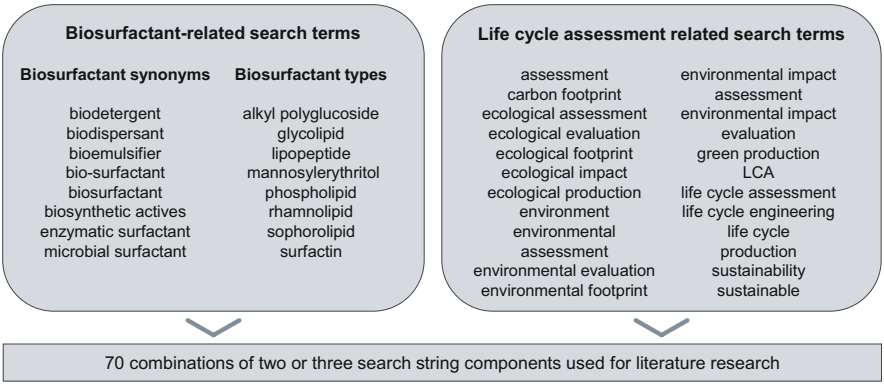


Fig. 1 Search string combinations used during the literature research

2.2 Definition of Relevance Criteria

As this literature review focuses on the evaluation of environmental impacts in a holistic way, publications were considered if an LCA was conducted. Furthermore, only peer-reviewed articles were taken into account to ensure a high-quality level. All types of microbial biosurfactants were included. During the research it became clear that the very narrow definition of biosurfactant limited to the microbial pathway would exclude some detailed LCA studies of alkyl polyglycosides (APGs), which are chemically produced but bio-based surfactants and often referred to as "first generation biosurfactants" [37]. Therefore, we included these studies in this review.

2.3 Screening Procedure

An illustration of the screening procedure is given in Fig. 2. First, if the search string produced a high number of results (more than 200 results), the search string was

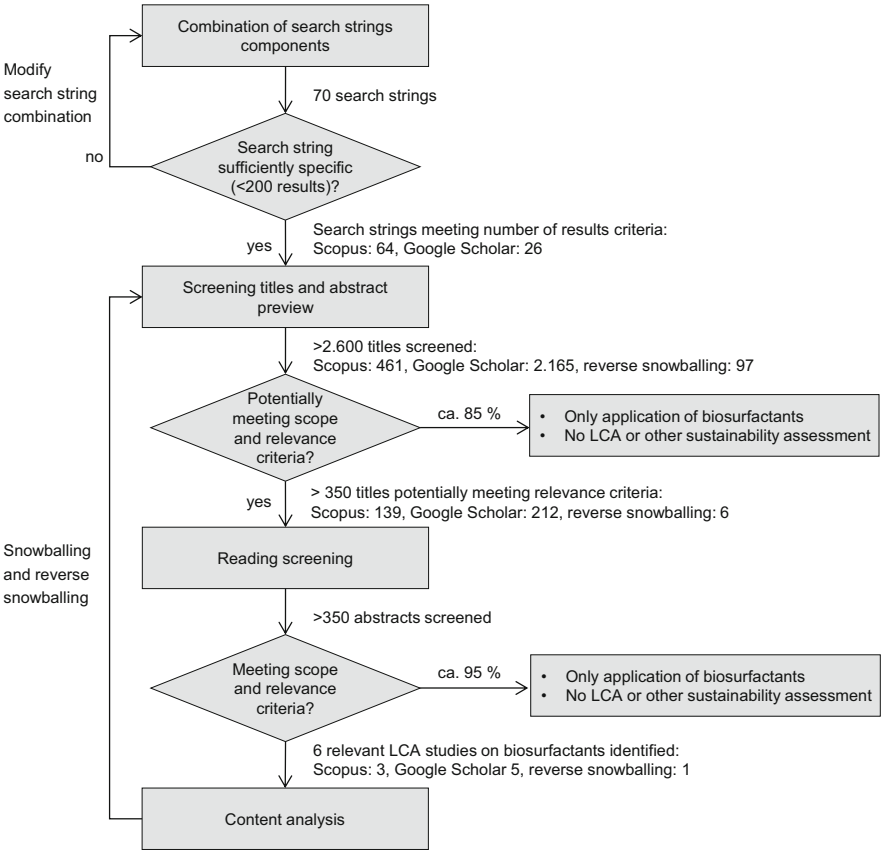


Fig. 2 Screening procedure during the systematic literature research

assumed to be too unspecific. In this case some results were screened to verify the low share of relevant studies, and the search string was modified to increase the share of relevant literature. Second, all titles and abstract previews of sufficiently specific searches were screened to examine if the study could be relevant to the scope of this literature review. Third, the next step was to read the full abstract and check against the predefined relevance criteria. Additionally, more relevant articles in the field of interest were retrieved from the relevant studies' citations (snowballing) and using the Scopus online database citation tracking (reverse snowballing).

2.4 Content Analysis Approach

Finally, the identified studies were analyzed in detail. For this content analysis, the most important LCA characteristics were defined. These LCA characteristics include:

- biosurfactant type,
- goal and scope,
- functional unit,
- software and database used,
- chosen impact assessment characterization method and categories,
- raw materials,
- production scale and production pathways,
- the field of the surfactant's application,
- regional and temporal context.

These characteristics were analyzed for each LCA study on biosurfactants, compared to the other LCA studies' characteristics and discussed.

3 Results

This section first provides a general overview of the findings during the research for this publication. Next to numerous studies related to the application of biosurfactants, two related topics to LCA in the general field of environmental analyses were identified and a short summary is given for both ecotoxicity and biodegradability. Finally, an overview is presented for the six identified LCA studies of biosurfactants.

3.1 General Overview Over the Search Findings

The titles of more than 2,600 documents were screened within this literature research, consisting of almost 500 search results from Scopus and more than 2,100

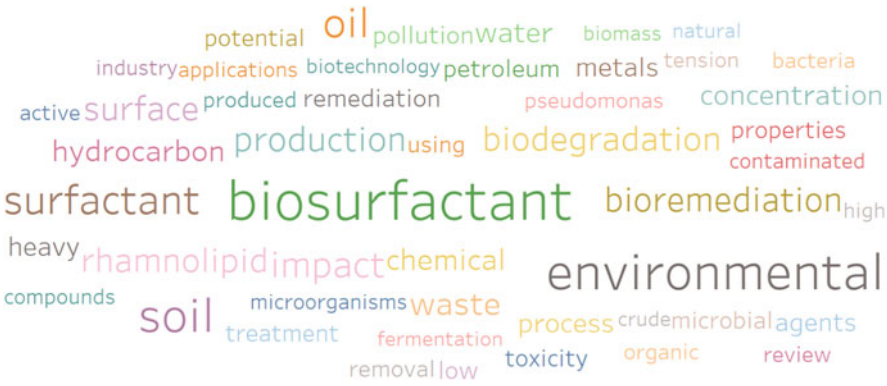


Fig. 3 Word cloud of the top 50 words from abstract and keywords of all results in an exemplary search string combination of a Scopus search: "biosurfactant" AND "environmental impact" (71 results)

search results from Google Scholar. Most of the literature from the first evaluation step covers related subjects rather than assessing the environmental sustainability of the surfactants. Articles in this context focusing solely on the application of biosurfactants account for a large share of results for the used search string combinations. The potential application of biosurfactants for remediation is widely discussed in academic literature [38]. Documents focusing on remediation account for approximately half of the overall search findings.

To illustrate our findings, we created a word cloud of the 50 most frequently occurring words in title, abstract, and keywords of the 71 documents found on Scopus using one of the search string combinations, that was specific enough but still resulted in articles from a broad range of fields ("biosurfactant" AND "environmental impact"), displayed in Fig. 3. The list of words was processed by only including the most frequently used words linked to the content of the text, i.e. excluding words without meaning, such as articles, conjunctions, and pronouns. In a second processing step, words found in singular and plural were aggregated to a single word. The frequency of each word is represented by the size of the word in the illustration, with "biosurfactant" having the highest number (267) and "compounds" the lowest (34). This visualization in Fig. 3 shows many remediation-related words, such as "bioremediation," "biodegradation," "pollution," and "removal." Various research groups reported on biosurfactant-enhanced bioremediation processes [38, 39]. Biosurfactants can be used in remediation of oil, petroleum, and diesel spills [40], polycyclic aromatic hydrocarbons [41, 42], and heavy metal contaminations of soil and ground water [43, 44]. The application of biosurfactants increases the solubility of hydrophobic substances and bioavailability for microorganisms. Corresponding word cloud listings are, for instance, "heavy," "metal," and "soil."

Next to the application potential of biosurfactants in bioremediation, a further research field is the use of biosurfactants in tertiary oil recovery discussed in various articles [45–47]. It is represented by roughly 20% of documents in the search results. Furthermore, main applications for biosurfactants are represented in the search findings, for example, as detergents in washing agents and household cleaning

products or as emulsifiers in cosmetics [48, 49]. Furthermore, researchers are investigating anti-bacterial, anti-fungal, and anti-cancer properties of biosurfactants, which offers opportunities for the use in medicine in the future [18, 50].

Another large share of results deals with new and optimized production processes for biosurfactants (corresponding words in Fig. 3: "process," "fermentation") and their substance properties ("properties," "tension"). The use of microorganisms (corresponding words in Fig. 3: "bacteria," "biomass," "fermentation," "microorganisms," and "pseudomonas") as essential feature of biosurfactants is often put in contrast to the conventional production pathway for surfactants ("chemical"). Several articles focus on process optimization to improve the yield and conversion rate through enhanced production strains, feedstocks, and operating conditions [23, 51–53]. The use of agricultural side products and waste streams as feedstock for the biosurfactant production offers potential for cost reduction (corresponding word in Fig. 3: "waste").

For the next selection, over 350 articles were considered potentially relevant. This accounts for approximately 15% of the search results. Due to the information provided in the abstract, six articles met the predefined relevance criteria investigating the environmental impact of biosurfactants with an LCA approach.

3.2 Ecotoxicity of Biosurfactants: A Summary

Biosurfactants are described to exhibit a "low toxicity" respectively "lower toxicity" compared to fossil-based surfactants [24, 25]. However, only few toxicity studies on specific biosurfactant variants deploying bioassays are available [26–29]. Therefore, by increasingly applying biosurfactants, a probable entry into the environment has to be considered and toxicity carefully assessed [26]. Different toxicity impacts ranging from the environment (ecotoxicity, e.g., phytotoxicity, aquatic or microbial toxicity) to humans (e.g., general toxicity, reproductive toxicity and carcinogenicity) are conceivable given the above-mentioned applications [54, 55]. In the scope of this short summary, we are focusing on ecotoxicity given that biosurfactants may be used as household detergents and potentially ending up in the environment. Ecotoxicity has been described as a chemical compounds' "[. . .] toxic impact to the organisms living in an environmental compartment like water, sediment or soil, is a substance-specific property forming an essential element for the environmental safety assessment" [55]. It is emphasized that not only the toxicity of a substance has to be evaluated but also whether the amount released into the environment is sufficiently high to have a toxic impact. This classification is underlined by the environmental risk assessment regulation "REACH" as outlined by the European Chemicals Agency, ECHA. For risk characterization of a substance, they describe a ratio of (predicted) environmental concentration (PEC) to the (predicted) no-effect concentration (PNEC) [55, 56]. In the following an exemplary study from 2016 was selected to illustrate the assays deployed to test ecotoxicity of a (bio)surfactant. The authors Johan et al. tested the toxicity of mono-rhamnolipid (m-RL) produced by a recombinant *Pseudomonas putida* strain using different representative

bioindicators. Different model organisms were treated with a dilution series of m-RL. The organisms were used as bioindicators to test for: (1) embryotoxicity and teratogenicity (*Danio rerio*), (2) cytotoxicity and growth inhibition (*Aspergillus niger, Candida albicans*) as well as (3) immobilization (*Daphnia magna*) according to OECD guidelines, international DIN standard and reported by [57–60]. Toxicity was described by means of LC_{50} respectively EC_{50} values which are the lethal concentration of a substance causing death to 50% of the organisms exposed respectively the effect concentration causing 50% of immobilization of the organisms exposed. High values represent a lower toxicity. The authors reported a mean LC_{50} value of 60 mg/L m-RL for *D. rerio*, a dose dependent decrease in growth for *C. candida* between 17 and 51 g/L, prevention of hyphen formation of *A. niger* at 68 g/L and an EC_{50} value of 50 mg/L (after 24 h) respectively 30 mg/L (after 48 h) for immobilization of *D. magna*. In Table 1 representative (eco)toxicity studies for prominent biosurfactants and synthetic surfactants are listed for comparison.

Table 1 Toxicity studies for biosurfactants. LC_{50} (50% of exposed organisms killed) and EC_{50} (50% of exposed organisms immobilized) with respective time of exposure to substance (rounded values)

Surfactant	Bioindicator	LC_{50} (1)/EC_{50} (2)	Reference
Biosurfactants			
Mono-RL	*D. rerio*	60 mg/L (1; 48 h)	[26]
	C. albicans	>17 g/L, growth inhibition	
	A. niger	68 g/L, prevention hyphen formation	
	D. magna	50–30 mg/L (2; 24–48 h)	
RL and sophorolipids	"Aquatic toxicity"	20–77 mg/L (2; N/A)	[29, 61, 62]
Surfactin	*D. magna*	170 mg/L (2; 48 h)	[28]
Saponin	*D. magna*	128 mg/L (2; 48 h)	[28]
Sophorolipids	*D. magna*	11 mg/L (NOEC[a])	[29]
Sophorolipids	"Aquatic toxicity"	29 mg/L (2; 48 h)	[37, 63]
Synthetic surfactants			
Sodium dodecyl sulfate	*D. rerio*	4 mg/L (1; 96 h)	[64]
	D. magna	24–29 mg/L (2; 24 h); 18 mg/L (2, 48 h)	[65, 66]
LAS ($C_9 - C_{14}$)[b]	*D. magna*	53–0.7 mg/L (2; 48 h)	[67]
LAS ($C_{10} - C_{18}$)[b]	*D. magna*	30–0.1 mg/L (1; 48 h)	[68]
LAE ($C_{14}AE_1 - C_{14}AE_9$)[c]		0.8–10 mg/L (1; 48 h)	
Alkyl polyglycoside (C_8)	*D. magna*	557 mg/L (2, 48 h)	[69]
Alkyl polyglycoside (C_{12-14})		12 mg/L (2; 48 h)	
Triton X-100	*D. magna*	18–26 mg/L (2; 48 h)	[70]

[a]NOEC, no observed-effect concentration
[b]LAS, linear alkylbenzene sulfonates
[c]LAE, linear alkyl ethoxylate ($CH_3 - (CH_2)X - (C_2H_4O)YH$); X = 13, Y = 1–9

3.2.1 Toxicity in LCA

The impact assessment part of an LCA can include toxicity related impact categories, such as fresh-water ecotoxicity or human toxicity. Generally, the scope of an LCA study includes the entire product system. Consequently, all substances entering and leaving the product system (inputs and outputs) are taken into account. For the evaluation of the environmental impacts, each substance of the inventory is taken into account by multiplying the emitted amount with the respective characterization factor [31, 32]. The development of characterization factors is complex and specific for each impact category. Characterization models include the fate of a substance once it is emitted into a compartment of the environment (air, water, soil), the exposure of organisms (e.g., humans) to this substance and its effect once an organism has been exposed [71, 72]. Especially for toxicity related impact categories, various differing models are available and calculations are bound to uncertainties [72, 73]. For example, a recent study investigated the availability of characterization factors for pharmaceutical emissions and their modelling in LCA. The results of the study revealed several relevant methodological gaps, such as missing specific impact pathways and a limited availability of characterization factors in existing toxicity models regarding the studied substances [74].

Therefore, the toxicity of biosurfactants as investigated in the above-mentioned studies could be included in future LCA studies, if proper characterization factors are developed. At the same time, evaluating toxicity categories in LCA can shed light on the impacts of the overall product system in this regard, while keeping in mind the inherent uncertainties of current modelling approaches. However, LCA cannot be used to determine the toxicity of a biosurfactant, or any substance for that matter.

3.3 Biodegradability of Biosurfactants: A Summary

As indicated by our search findings, biodegradability is a characteristic often associated with biosurfactants. However, studies that actually assess their biodegradability are rather limited and were previously described by Klosowska-Chomiczewska et al. [75]. At the same time antimicrobial characteristics are also attributed to some biosurfactants [16, 47, 76–80]. While this seems promising for their application in pharmaceuticals or as antimicrobial agents, it may pose a challenge for biodegradation at high biosurfactant concentrations. After their release to the environment, these antimicrobial characteristics of the biosurfactants could prevent or at least delay a biodegradation [78]. Some described applications include an intended emission of biosurfactants to the environment during their use phase. One example is the widely described application for bioremediation, where their amphiphilic characteristics are used to facilitate the solubilization of hydrocarbons and thus enable their subsequent biodegradation [24, 47, 81]. Here the biodegradability of the used biosurfactant itself would be a necessary prerequisite for its application, as it

stays in the environment. Another example is using biosurfactants as detergents in cleaning agents or as ingredients for cosmetics [82]. After their use by consumers and disposal into the public wastewater system, an exposure to a water treatment plant and afterwards the environment occurs.

The consideration of biodegradability is already taken into account in many regulations and laws. The European Detergent Regulation (EC) No 648/2004, or even REACH, imposes minimal degradation limits for a surfactant to be approved for application as a detergent or as a chemical ingredient in other products, thus making biodegradability assessments inevitable.

In biodegradability tests, a mineral medium solution or suspension containing the test substance is inoculated with a mixed population of microorganisms (derived from activated sludge, sewage effluents (unchlorinated), surface waters and soils, or from a mixture of these [83] and incubated under aerobic or anaerobic conditions, while dissolved organic carbon (DOC), CO_2 production, or O_2 uptake are measured as indicators for biodegradation. A widely used test method for biodegradability is standardized by the Organisation for Economic Co-operation and Development (OECD). According to the OECD guideline, a substance is considered "readily biodegradable," when 70% of DOC, or 60% of the theoretical CO_2 (equivalent to C content in the test substance) is measured within a 10-day window in the 28-day test period under test conditions [83].

As published in part by Klosowska-Chomiczewska et al. [16] and summarized in Table 2, most examined microbial biosurfactants show biodegradability and in some cases with rates superior to some representative synthetic surfactants. Variations in biodegradability results amongst the same biosurfactant group can be attributed to either different test conditions or different compositions of the used biosurfactant mixtures. For better comparability it is thus advised to use the standardized OECD methods for future biodegradability studies.

3.3.1 Biodegradability in LCA

Information on the biodegradability of the biosurfactant is necessary for end-of-life modelling in LCA, especially when assessing the ecotoxicity of a certain biosurfactant after its release to the environment, as discussed above. Biodegradability values would provide the data needed to model the impact of remaining residues of the biosurfactant after its use phase, whether the release to the environment is during the use phase (e.g., for bioremediation) or rather after its use phase (e.g., as an ingredient in detergents or cosmetics).

As shown in our short summary, most examined biosurfactants show ready biodegradability. This means that $\geq$60% of the biosurfactant is biodegraded within a 10-day window, which is a good indication for an overall low impact on ecotoxicity. However for a proper assessment of ecotoxicity, detailed kinetics of biodegradation should be considered.

Table 2 Biodegradability tests on microbial biosurfactants and synthetic surfactants in comparison. A substance is considered readily biodegradable if ≥60% is biodegraded within 10 days of the test period and indicated with a "+" in the table. If the presented data is not sufficient to determine ready biodegradability, this was indicated with N/A

Surfactant	Producing microorganism	Test used (OECD/ other)	Readily biodegradable	Reduction of surfactant concentration (duration needed)	Reference
Microbial biosurfactants					
Surfactin, iturin, fengycin	*Bacillus subtilis*	Other	+	68.3% (166 h)	[84]
Surfactin	*Bacillus subtilis*	Other	+	69.1% (166 h)	[84]
Flavolipids	*Flavobacterium sp.*	Other	*N/A*	42.5% (166 h)	[84]
Arthrofactin	*Arthrobacter oxydans*	Other	+	73.4% (166 h)	[84]
Trehalolipid	*Dietzia maris*	Other	+	59.6% (166 h)	[84]
Surfactin	*Bacillus subtilis*	Other	+	>65% (72 h) at concentrations <100 mg/L	[28]
Surfactin	*Bacillus subtilis*	OECD 301 E	+	>60% (9 days)	[28]
Surfactin	*Bacillus subtilis* HSO121	Other	+	>60% (<3 days)	[82]
Exopolysaccharide EPS_{2003}	*Acinetobacter calcoaceticus* CBS 962.97	Other	+	90% (120 h)	[85]
Rhamnolipid	n.a.	Other	+	>60% (4 days)	[86]
Mono-Rhamnolipid	*Pseudomonas aeruginosa* ATCC 9027	Other	+	>60% (approx. 8 days)	[86]
Sophorolipid	*Starmerellabombicola*	OECD 301C	+	>60% (8 days)	[87]
Mannosylerythritol lipid	*Moesziomyces antarcticus* KCTC 7,804	Other	N/A	N/A	[88]
Mannosylerythritol lipid	n.a.	OECD 301B	+	64% (10 days)	[89]
Synthetic surfactants					
SDS (sodium dodecyl sulfate)	–	Other	N/A	24.8% (166 h)	[84]

LAS (linear alkyl sulfates)	–	OECD 301D	+	>60% (7 days)	[90]
Palm based MES C12 (methyl ester sulfonates)	–	OECD 301D	+	>60% (5 days)	[90]
Various alkyl polyglycosides	–	Modified, based on OECD	+	>70% of DOC (10 days)	[91]

3.4 Systematic Analysis of Existing LCA Studies

A total of six articles were found as described above. To illustrate the specific focus of these studies, we created a second word cloud shown in Fig. 4 with the top 50 words from title, abstract, and keywords of the identified relevant LCA studies for this review. Therefore, Fig. 4 visualizes keywords in a more specific context compared to Fig. 3. Out of the top 50 words for each visualization, 13 are the same, for example "environmental" and "biosurfactant" are similarly prominent compared to Fig. 3. Moreover, both Figs. 3 and 4 show terms related to the production of biosurfactants, production organisms, and biosurfactant types. However, the surrounding words in Fig. 4 show the specific focus of these studies, such as "life," "cycle," "impact," and "assessment." Very specific LCA related terms can be found, such as "functional," "unit," and "global," "warming," "potential," or "CO_2". Furthermore, each study left a distinct "finger print," one example being the representation of *Azotobacter vinelandii*, a production strain used in one of the studies [52]. The number of occurrence for each word in Figs. 3 and 4 can be found in the supporting information.

In the following, the content of all articles is summarized and divided into two sections. One dedicated to the three articles covering LCA studies of APGs and the other dedicated to LCA studies of microbial biosurfactants. Table 3 provides an overview over all 6 investigated articles and the specific boundary conditions of the conducted LCAs.

3.4.1 LCA Studies of APGs

Three LCA articles of alkyl polyglycosides (APGs) were found. APGs are chemically derived from renewable resources, mainly tropical oils and a sugar fraction. As

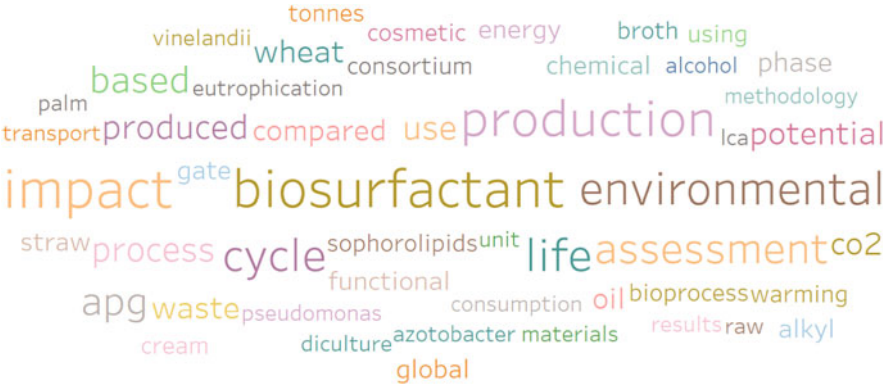


Fig. 4 Word cloud of the top 50 words from title, abstract, and keywords of the six relevant LCA studies on biosurfactants

Table 3 Overview of the characteristics of the investigated LCA studies of alkyl polyglycosides and microbial biosurfactants

	LCA studies of alkyl polyglycosides (APGs)			LCA studies of microbial biosurfactants		
Characteristics	Guilbot et al. [92]	Lokesh et al. [53]	Brière et al. [94]	Baccile et al. [37]	Aru and Ikechukwu [52]	Kopsahelis et al. [51]
Surfactant type	Alkyl polyglycosides	Alkyl polyglycosides	Alkyl polyglycosides	Sophorolipids, mainly acidic form	N/A	Sophorolipids (SL) and rhamnolipids (RL)
Raw materials	C16/18 fatty alcohol from transesterification of palm kernel oil (Malaysia, Indonesia), glucose from wheat starch	Wheat straw based octacosanol (supercritical CO_2 extraction) and levoglucosan (pyrolysis) Reference scenario: Palm kernel oil, glucose from wheat starch according to Guilbot et al.	Wheat straw, palm kernel oil; scenarios for use of glucose from corn starch and other fatty alcohol feedstocks; reference scenario: Palm kernel oil, glucose from wheat starch according to Guilbot et al.	Glucose, canola oil	Mineral oil wastes	SL: waste oil, glucose RL: waste oil
Scale	Industrial	Upscaling from laboratory process	Laboratory process	Industrial, upscaling from 150 L/batch pilot plant	Upscaling from laboratory process	Pilot
Application	Emulsifier in cosmetic formulation	N/A	N/A	Hand wash detergent	Remediation of crude oil spills	N/A
Geographic reference	France	Great Britain	France	Belgium	N/A	Greece
Scope of LCA	(a) Cradle-to-grave (b) Cradle-to-gate	Cradle-to-gate	Cradle-to-gate	Cradle-to-grave	Gate-to-gate	Gate-to-gate

(continued)

Table 3 (continued)

Characteristics	LCA studies of alkyl polyglycosides (APGs)			LCA studies of microbial biosurfactants		
	Guilbot et al. [92]	Lokesh et al. [53]	Brière et al. [94]	Baccile et al. [37]	Aru and Ikechukwu [52]	Kopsahelis et al. [51]
Functional unit	(a) Cosmetic oil in water emulsion for face hydration of a consumer during 1 year (b) 1 t packed APG	1 g APG	1 t APG	1 hand wash (2.3 g surfactant +0.64 L water	1,000 kg surfactant	1 kg surfactant
LCA software	SimaPro 7.3.2	Spreadsheet calculation	SimaPro 8.3	N/A	N/A	SimaPro 7.3.3
LCI database	Ecoinvent	Ecoinvent 2.2, Biograce 2013, Defra 2015	Ecoinvent 3.3	Ecoinvent 3.1	N/A	Ecoinvent 2.0
Data sources	Industrial primary data from industry (transesterification, deacetylation) and literature (raw materials, use phase)	Literature	Literature + experimental data	Experimental data (pilot scale, 150 L)	Experimental data, simulation of upscaling to industrial	Experimental data + literature
LCIA methods	Impact 2000+, Eco Indicator 1999, IPCC 2007 GWP 100 a, Ecoinvent	DEFRA, IPCC	ReCiPe 2016 midpoint	ILCD midpoint v1.04, ReCiPe endpoint (Europe H/A) v1.10	N/A	EPD 2008 V1.03
Impact category	Global warming, mineral resources, petrochemical	Global warming potential from direct emissions, global	Climate change, ozone depletion, ionizing radiation, fine	*Midpoint:* climate change, ozone depletion, human toxicity,	Global warming potential, acidification potential,	Global warming potential, ozone layer depletion,

	resources, ecotoxicity, acidification, eutrophication, water consumption	warming potential from land use, fossil-derived energy footprint, water consumption waste-factor	particulate matter formation, photochemical oxidation formation (eco-system quality, human health), terrestrial acidification, fresh-water eutrophication, human toxicity (cancer, non-cancer), ecotoxicity (terrestrial, fresh-water, marine), land use, water use, mineral and fossil resource scarcity	particulate matter, ionizing radiation, photochemical ozone formation, acidification, eutrophication, ecotoxicity, land use and resource depletion *Endpoint:* damage to human health, ecosystems and resources	eutrophication potential	photochemical oxidation, acidification potential, eutrophication, non-renewable fossil energy demand

APGs have been commercialized in large quantities for many years, studies on their characteristics and applications are available as well as LCA studies. For this reason, these studies can serve as a benchmark for LCAs of other biosurfactants. Figure 5 illustrates the life cycle stages considered in the relevant LCA studies on APGs.

In 2013 Guilbot et al. conducted a cradle-to-grave life cycle assessment for an APG containing cosmetic cream and a cradle-to-gate life cycle assessment for raw APG [92]. The APGs are synthesized via Fischer's glycosylation of fatty alcohols from palm kernel oil and glucose in France. The data for the synthesis of APG is based on industrial primary data. Palm trees are assumed to be cultivated in Malaysia and Indonesia, as well as the oil extraction. The following conversion of raw palm kernel oil to fatty alcohol via transesterification and hydrogenation in Germany is also based on industrial primary data. For the saccharide feedstock, glucose is assumed to be produced from wheat starch in France. The LCA was carried out using SimaPro 7.3.2 software and the Ecoinvent 2.0 database for background data. The functional unit for the first investigation was defined as 1,000 kg of packed APG. In addition, the use of the above-mentioned APG as emulsifier in a cosmetic cream was investigated in a cradle-to-grave LCA. In this scenario, the functional unit was defined as the provision of a cosmetic face hydration cream for one person for a year, which equals 584.0 g of cream containing 29.2 g of APG. In the formulation process, water, an oil phase, and APG are mixed. Packaging, transportation, store supply, purchasing by the consumer, and use are also taken into account for this consideration. Guilbot et al. showed that for the cosmetic cream, the formulation phase and the use phase are most relevant for the environmental impact. The main impact in the formulation phase is caused by the oil used in the formulation. Regarding the use phase, transportation of the cream from stores to consumers plays an important role. By contrast, the production of APG does not contribute much to the environmental impact of the final cosmetic cream because of the low weight share of only 5% in the formulation. Focusing on the production of the APG, Guilbot et al. showed that raw materials, in particular fatty alcohol from palm kernel oil, account for the highest environmental impacts in all categories considered. The environmental impact of the fatty alcohol is induced through land use change in a great extent and largely depends on the cultivation conditions of palm trees. Uncertainties in the former land use, soil type, yield, and other assumptions more for the palm tree cultivation result in significant variations on the carbon footprint.

Lokesh et al. introduced a method to produce APGs from only wheat straw instead of typically used raffinated glucose and vegetable oils [53]. In this new production process, the feedstock chemicals for the APG synthesis, octacosanol and levoglucosan, are both produced from wheat straw. First, wax esters are produced via supercritical CO_2 extraction of wheat straw. In a fractionation process, octacosanol is separated from other fatty alcohols, fatty acids, esters, and dewaxed straw. In the next process step, dewaxed wheat straw is pelletized. In a low temperature microwave pyrolysis process with in-situ separation, the anhydro sugar levoglucosan can be produced from dewaxed straw pellets and used as feedstock for the glycosylation reaction for APG synthesis. The research group carried out an LCA to investigate if this new method is more advantageous to

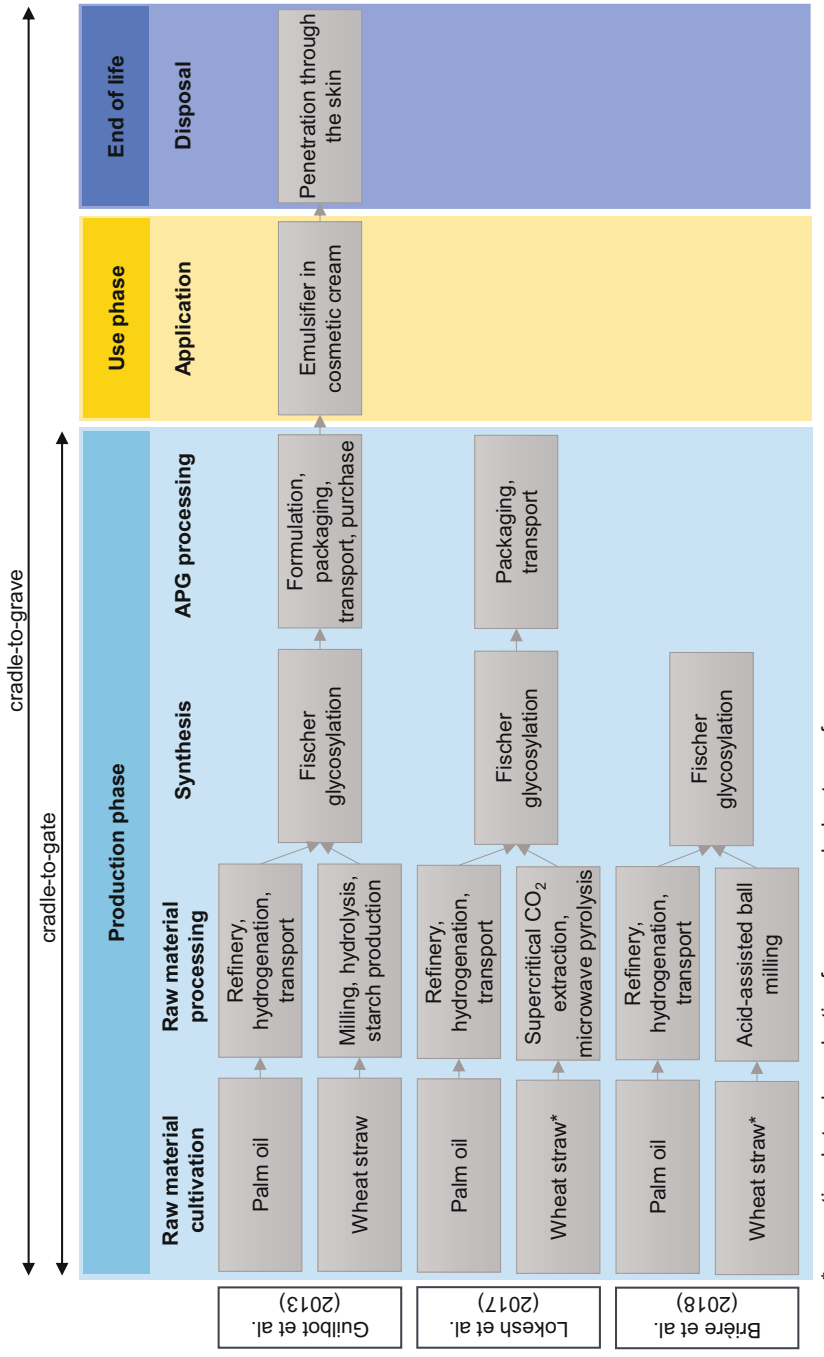


Fig. 5 Life cycle stages considered in the discussed LCA studies on APGs

conventional APG production from an environmental point of view. Therefore, a functional unit of 1 g APG was defined and the above-mentioned LCA model of Guilbot et al. was used as a baseline scenario. The APG production in the baseline scenario is characterized by the use of glucose from wheat starch and palm kernel oil instead of wheat straw. The cultivation of wheat and production was assumed to take place in Great Britain. The research group used different literature data sources, Ecoinvent 2.2 data, other databases and national inventories, as well as experimental data for a newly developed ball milling process. Environmental impacts were calculated by spreadsheet calculations for the impact category global warming potential and subdivided into direct emissions and emissions from land use. The wheat straw based APG appeared to have a lower direct GWP than the glucose and palm kernel oil based counterpart, for both direct emissions and land use emissions. The global warming potential caused by land use change was further investigated. The study found that the fatty alcohol and starch production caused a higher GWP than octacosanol and levoglucosan extracted from wheat straw. Furthermore, a carbon storage credit was given for biochar to the wheat straw based APG. Biochar is also known as activated wheat straw. It is formed as a side product of the pyrolysis process. CO_2 stored as biogenic carbon in the biochar could potentially be sequestered via re-incorporation of the biochar into the soil. Three different change scenarios were implemented to evaluate emissions from land use. Besides the LCA impact category GWP, the additional indicators fossil-derived energy consumption, water consumption, and the waste factor (mass of waste per mass of product) were calculated. In a later study, Lokesh et al. extended their sustainability analysis by conducting a life cycle costing analysis to quantify the economic feasibility and resource efficiency of the described production process of APG from wheat straw [93].

Another LCA study investigated the environmental impact of the APG production from wheat straw and fatty alcohols from various sources [94]. Although Brière et al. used the same raw materials for the APG production as Guilbot et al., the feedstock for the Fischer glycosylation reaction is produced from wheat straw via a newly developed acid-assisted ball milling process. Wheat straw crushing and depolymerization of cellulose and hemicellulose take place at the same time. The reactive short chain oligosaccharides obtained are directly used in the APG synthesis with fatty alcohols, without any separation or purification steps. A cradle-to-gate LCA was conducted for the production of 1,000 kg of APG to revise the environmental impact of this new pathway. The SimpaPro 8.3 software and the Ecoinvent 3.3 database were used and the production was assumed to take place in France. In the reference scenario, corn starch was used as carbohydrate source for the glycosylation instead. Fatty alcohols were assumed to be obtained from palm kernel oil in both scenarios. The impact assessment was carried out using the ReCiPe 2016 characterization factors and all 17 midpoint impact categories were taken into account. The research group found that using this new method, the environmental impact of the carbohydrate can be significantly reduced. The provision of fatty alcohols from palm kernel oil causes the largest share in almost all impact categories. Because of the low share of wheat straw and acid-assisted milling to the impact of

the APG production, allocation procedure and the electricity mix only have a low influence on the overall impact. Additionally, three different sources of fatty alcohols were compared to the global market mix of fatty alcohols used for the baseline scenarios, in particular palm kernel oil, coconut oil, and petrochemicals. In general, coconut oil generated the largest impacts, while palm kernel oil seems to generate lower impacts. In contrast to the low impacts of wheat straw on the environmental impact of the final product, the source of fatty alcohols strongly affects the APGs environmental performance.

Three LCA articles on the environmental impact of APGs, a first generation biosurfactant, were summarized in this section and an overview is provided in Table 3. The first study used primary data from industrial APG production (Guilbot et al.). The two subsequent articles originate from other research groups. They both used raw materials for the APG production obtained from wheat starch in newly developed processes. These two new APG production processes based on wheat straw were both investigated in laboratory experiments. The scaled-up experimental data was then used for a life cycle assessment analysis to identify hot spots in the production. Both studies on wheat straw based APG referenced the industrial data study of Guilbot et al. as a benchmark scenario.

3.4.2 LCA Studies of Microbial Biosurfactants

Following the defined relevance criteria of this literature review, only three LCA studies of microbial biosurfactants were found. The life cycle stages considered in these studies are presented in Fig. 6. Their scopes and main findings are shortly summarized and discussed in the following paragraphs.

In 2017, Baccile et al. presented a multidisciplinary study focusing on acetylated acidic sophorolipids [37]. The LCA study was conducted in addition to synthesis, purification and characterization of the produced compounds, which were carried out by all co-authors simultaneously using the same sample from one batch. For the LCA, a cradle-to-grave approach was chosen. The application of the surfactant in a household hand-washing detergent was investigated using the functional units of "1 hand wash" and, additionally, "1 kg of surfactant" for a comparative analysis. While the focus was put on the impacts from the experimental fermentation and purification processes of the original 150 L pilot scale, the processes were "assumed at a larger scale" for the calculations. The exact scale taken into account is not specified by the authors. The geographical reference is Belgium. The results for midpoint indicators (ILCD midpoint v1.04 method) showed that the highest impacts in all evaluated impact categories originate in the production phase, specifically from the substrates (glucose and rapeseed oil). While the use phase had overall small impacts, the end of life phase had noticeable impact in some categories, e.g. climate change and eutrophication. Additionally, using endpoint indicators (ReCiPe endpoint v1.10 Europe H/A) the authors compare their results with reference products ranging from linear alkylbenzene sulfonate (LAS) to APGs. The total environmental impacts of the investigated sophorolipids are in the same range as for the reference products.

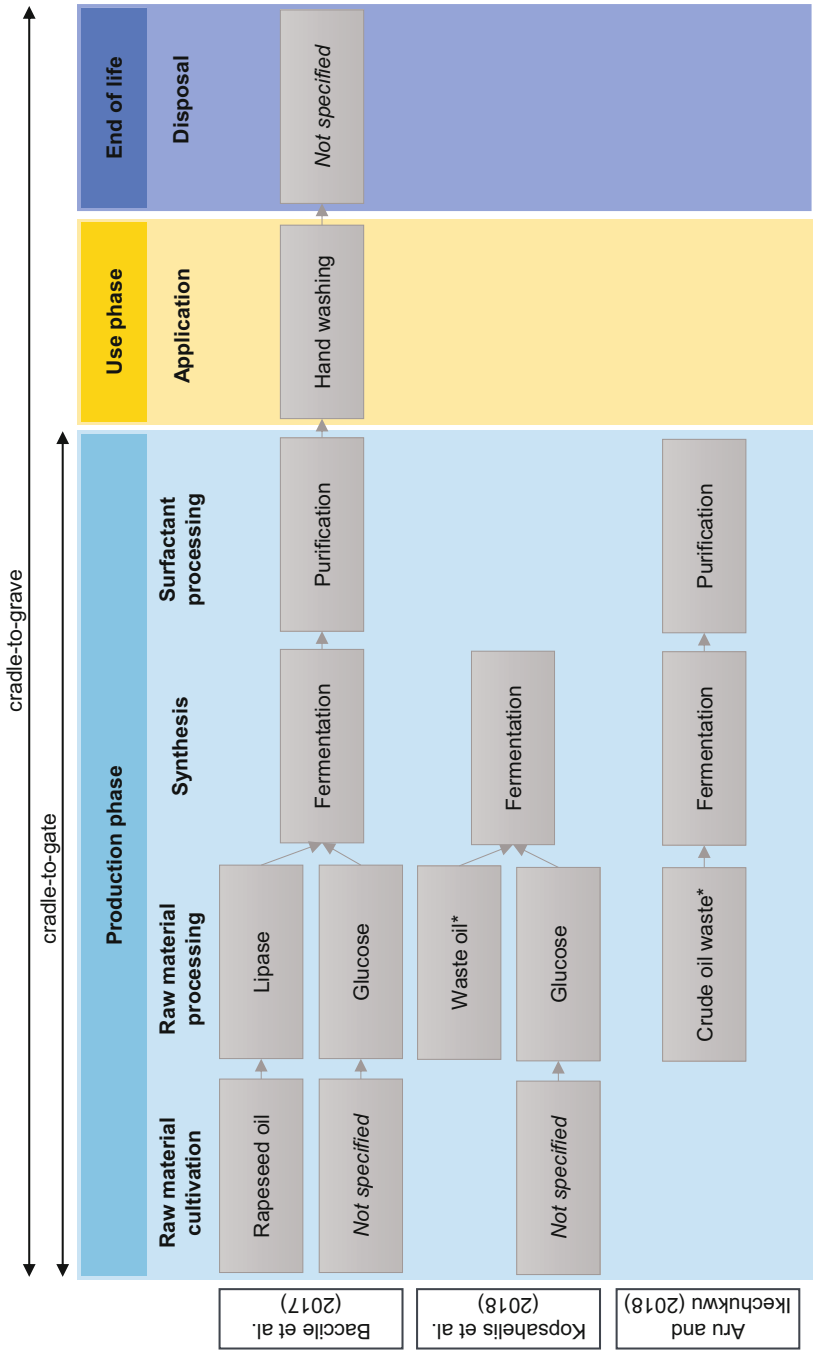


Fig. 6 Life cycle stages considered in the discussed LCA studies on microbial biosurfactants

It is pointed out that the currently small production volume should be kept in mind, as the results are shown per volume of product. Baccile et al. conclude that the environmental impacts depend largely on the raw material input and, therefore, an optimization of the substrate ratio would lead to significant improvements. Moreover, the use of second-generation raw materials is mentioned as another optimization possibility.

An article by Aru and Ikechukwu presents a gate-to gate LCA of biosurfactants produced by a diculture of *Azotobacter vinelandii* and *Pseudomonas* sp. for an application in bioremediation of oil spills in Nigeria [52]. The specific surfactant type is not mentioned in the article. The analysis was based on laboratory processes, including fermentation, purification steps, and recycling of solvents, and linearly scaled to industrial production of "1,000 kg surfactant" which served as functional unit. Although the LCI data for the entire process is given, only the metabolic CO_2 emissions from the cultivation process are taken into account for calculating the global warming potential. Similarly, the NH_3 emissions are solely used to calculate the acidification potential. Additionally, the electricity consumption is investigated separately. Emissions to the environment are calculated assuming a power supply by natural gas with a 33% conversion efficiency and using emission factors from the United States Environmental Protection Agency. The authors conclude that the emissions from the power supply contribute the most to the overall environmental impacts. Furthermore, they point out that the intended application plays a key role, as in the investigated case of bioremediation the microorganisms could be directly applied to the soil avoiding the production of large amounts of surfactant in a technical process and the associated emissions from the power supply.

Kopsahelis et al. [51] investigated the production of biosurfactants and bioplasticizers from waste oils within the EU-project Bio-SURFEST [51]. In this study, the environmental impacts resulting from the production of rhamnolipids and sophorolipids are analyzed in a gate-to-gate LCA. The fermentation process conditions of a pilot production in Greece and the reference year 2013 are taken into account. Since the study focused on the synthesis of biosurfactants, the functional unit is defined as "1 kg of product" and detailed results are provided for the pre-inoculum, inoculum, and fermentation process stages. Purification of the surfactants was not taken into account in this study. The results are calculated for six midpoint indicators using the EPD 2008 V1.03 method. The authors point out that the environmental impacts of the investigated rhamnolipids production are lower compared to those of the sophorolipids production, due to the shorter duration of the main fermentation process resulting in lower thermal energy and electricity demand as well as less CO_2 emissions from the metabolic activity of the microorganisms during fermentation. Furthermore, the obtained results are discussed in context with the findings of Baccile et al. [37] and Guilbot et al. [92] and found to reach similar conclusions. It is concluded that the biosurfactant fermentation mainly contributes to the overall environmental impacts. Furthermore, Kopsahelis et al. [51] point out that there are only few studies in recent literature that conducted life cycle based sustainability analyses and, consequently, their importance is highlighted.

In summary, the three analyzed articles presented LCA results of various microbial biosurfactants using experimental data. While the specific surfactant type was not mentioned in the limited study by Aru and Ikechukwu, Baccile et al. and Kopsahelis et al. investigated sophorolipids and rhamnolipids in detail from a life cycle perspective. While Kopsahelis et al. and Aru and Ikechukwu implemented a gate-to-gate approach, Baccile et al. evaluated the application of the studied biosurfactant in a hand-washing detergent and compared it with other conventional surfactants. Kopsahelis et al. point out that there is a lack of similar studies in current literature, which is reflected by the fact that Baccile et al. and Aru and Ikechukwu do not mention other studies in their articles.

4 Discussion of Findings in Published LCA Studies

Even though thousands of documents were screened and abundant literature on the characteristics and various environmental properties of biosurfactants are available, surprisingly only two detailed LCA articles of microbially produced surfactants could be found. As summarized above, ecotoxicity and biodegradability of biosurfactants are in the focus of researchers and these findings can be useful in the context of LCA. Contrarily, investigating the overall environmental impacts of these substances by means of LCA seems to be neglected so far in literature. Therefore, we broadened the scope of the original selection criteria during our research to include relevant LCA studies of other bio-based surfactants as well.

The studied surfactants in the investigated studies were microbially produced rhamnolipids and sophorolipids, as well as bio-based and chemically produced APGs. Only one study did not specify the biosurfactant type, although the microbial origin was made clear. It is not surprising that there were more studies found on APGs (half of the results), due to the fact that they have been commercialized on a larger scale. This is also reflected by the fact that the only study using industrial data for the LCA was Guilbot et al. [92] investigating APGs. All other studies used experimental or pilot-scale data and assumptions or simulations for scale-up calculations. In the case of the microbially produced biosurfactants, this reflects the fact that many biosurfactants are still being researched and developed in lab-scale. Similarly, only half of the investigated studies took into account the intended application of the surfactant. Furthermore, the applications mentioned vary from the use in a cosmetic cream or a hand-washing detergent to bioremediation.

Most of the analyzed articles focused on the biosurfactant production process, more specifically, in the case of microbially produced biosurfactants, on the fermentation process. While the downstream recovery and purification processes of the raw surfactant from the fermentation broth were not explicitly mentioned in Kopsahelis et al. [51], Baccile et al. described their ultrafiltration and extraction processes, but did not discuss the influence on the LCA results in detail [37]. This might be due to the fact that, in this case, “90% of the impact from the production phase is caused by the fermentation and [. . .] especially the use of the renewable resources” [37].

Generally, bio-based raw materials were in the focus of all studies and related uncertainties discussed, especially concerning tropical oils used for the production of APGs. In this context, the integration or omission of emissions from land use change has a high influence on the LCA results, as shown in the investigated studies. Furthermore, other associated topics, such as the loss of biodiversity, are relevant when comparing the environmental impacts of different types of surfactants. Due to these complex problems associated with tropical oils, waste streams are considered a more sustainable alternative. This fact is reflected by half of the studies: two studies that investigated wheat straw as a raw material for APG production and one study investigating waste oil for rhamnolipid and sophorolipid production.

Almost all studies were published in the last few years (2017 and 2018) showing the recent development of this research field. The exception of the earlier study by Guilbot et al. [92] became a sort of benchmark study. Another common attribute of almost all studies is their geographical reference. All studies clearly stating the location mentioned European countries (France, Greece, Belgium and Great Britain) and took this into account for the LCA modelling. Although Aru and Ikechukwu [52] mention Nigeria as the location of the intended application of their biosurfactant, it is unclear if this fact was taken into account for the LCA modelling.

The software applied to conduct LCA in three of the investigated studies was SimaPro, and therefore the most frequently used software in the investigated LCA studies of biosurfactants. One other study was carried out using spreadsheet calculations due to limitations of commercially available LCA software [53]. For two studies the used software was not further specified. The Ecoinvent database provided life cycle inventories for the background system for the majority of the investigated studies, except for one study where software and database were not specified.

A wide variety of impact assessment methods were used in the investigated studies, such as ReCiPe, ILCD, Impact 2000+, Eco Indicator, and EPD characterization factors for impact assessment at midpoint level. Baccile et al. additionally assessed environmental impacts at endpoint level based on weighted sums of midpoint impacts and used these to compare their results to a number of other surfactants. The results are given as absolute values in some studies, whereas several studies give the relative contribution of particular process steps to the overall impact without going into detail. This might be caused by the experimental character of the production process for microbial biosurfactants. At this early development stage, LCA is an excellent tool to identify the optimization potentials of the newly developed process. Thus, environmental hotspots can be found and improved before going into scale-up. The functional unit was defined considerably consistent by mass unit of surfactant (e.g., "1,000 kg of surfactant") in the investigated studies. Since the application of the surfactant was not specified in most cases, biosurfactants might be used in different products. Therefore, the function of a product is hard to predict and most studies' scope was defined as cradle-to-gate or gate-to-gate. Only two cradle-to-grave LCA studies were available. In these two cases, the quantification of the function of the biosurfactant containing product was possible. While Guilbot et al. took a closer look at the use of APG as emulsifier in cosmetic cream, which

represents a commercial use case of APGs already, Baccile et al. considered sophorolipids in a hand-washing detergent application.

Some studies concluded that the number of LCA studies of biosurfactants is limited, which is also in line with our findings. Furthermore, only one study compared their results to other surfactants. Baccile et al. showed that already small-scale production conditions (i.e., estimated upscaling from 150 L pilot scale) resulted in similar impacts compared to conventional detergents as reference products, such as bio-based soap, fossil-derived linear alkylbenzene sulfonate and oleochemically produced ethoxylated alcohol among others [37]. Future optimization seems likely and the authors mention the use of second-generation raw materials and increased efficiency of the production process in this regard. This fact can only encourage more researchers to conduct and publish LCA studies in the future.

The presented work provides a detailed overview of the published LCA studies on microbial biosurfactants based on a systematic literature research. Nevertheless there are still some limitations regarding the comparability of the investigated studies and the derivation of significant findings or recommendations, for example regarding a comparison of microbial biosurfactants with fossil-derived surfactants. In order to facilitate such a comparison, firstly, goal and scope of the LCA studies require a high level of similarity. The definition of the functional unit and cut-off criteria are crucial determinants for the LCA. Moreover, the selection of life cycle inventory databases for background processes and the impact assessment methods applied strongly affect the results of the impact assessment. Besides methodological aspects of the life cycle assessment, the development stages and production scales of microbial surfactants differ strongly from the production of conventional surfactants and, therefore, offer limited comparability. However, a study of conventional surfactants performed within the ERASM SLE project [34] provides a proficient overview on life cycle assessment of conventional surfactants and their precursors, of which some are also relevant for microbial surfactant production. Furthermore, Rebello et al. compare the outcomes of LCA studies of specific conventional surfactants and biosurfactants and highlight the relevance of LCA for environmentally friendly surfactant production [95]. The article finds that LCA of surfactants require the inclusion of appropriate impact factors and points out the need for suitable and consistent data as well as LCA expertise to perform a reliable comparison of different surfactants [95].

All in all, to perform a comprehensive LCA, it is required to take into account all life cycle stages, such as production, use phase, and disposal. Due to the early stage of their development, it is currently difficult to specify the use of microbial biosurfactants in final products and their corresponding disposal pathways and, therefore, to assess the use and end-of-life phases in LCA. Additionally, it is necessary for a comprehensive LCA to address a wide range of environmental impacts by assessing various impact categories in the impact assessment. This is essential to identify hotspots in the product life cycle, since a specific production step might correspond with high impacts in one impact category, while other production steps cause significant impacts in others. This way, a shift of burdens between impact categories and life cycle stages can be identified.

5 Conclusions and Research Perspectives

This study provided a detailed overview of existing LCA studies of biosurfactants by means of a systematic literature research. Over 2,500 documents were screened in this process leading to six published peer-reviewed LCA studies that were investigated and summarized in detail in this work. During the search process it became clear that biosurfactants are often considered to be environmentally friendly, due to the use of natural resources, their low ecotoxicity, and high biodegradability. These properties are taken into account when conducting LCA. However, they are not standalone indicators for sustainable products, but rather input parameters for a comprehensive sustainability assessment. Studies that exclusively investigated ecotoxicity or biodegradability of biosurfactants were not the primary focus for this review, though playing an important role for assessing environmental impact of biosurfactants. For this reason, ecotoxicity and biodegradability of biosurfactants were identified as relevant topics and discussed in short summaries as part of this work.

Regarding LCA studies of biosurfactants it can be concluded that the number of available literature is very limited. Furthermore, a comparison of the results of the investigated studies was not possible, due to various above-mentioned reasons. First, the scope of the studies varied from gate-to-gate to cradle-to-grave. Second, partial comparisons were not possible, because the results were calculated using various methods and only in some cases presented in absolute values. Third, the applications varied widely adding to the difficulty of a reasonable comparison. Taking the application into account is highly relevant to be able to make concrete statements about the environmental effects of a specific surfactant. Nevertheless, it became clear that the main influence on the environmental impacts in the production phase of the investigated biosurfactants can be attributed to the raw material inputs and energy demand during the fermentation processes.

Although only few types of biosurfactants were in the focus of the investigated studies, the overall importance of second- and third-generation feedstock for their production became clear. Especially in the context of APGs, the replacement of tropical oils, which are associated with burdens caused by emissions from land use change and the loss of biodiversity, can be recommended from an environmental point of view.

To the best of the authors' knowledge, this review is the first to provide a systematic and detailed overview of LCA studies of biosurfactants. At the same time, only two detailed studies of microbially produced surfactants could be found. Consequently, the need for implementing more LCA studies becomes clear. The findings of the investigated studies give promising insights into lab-scale processes. While individual environmental hotspots could be identified, the need for more transparency and detailed reporting of LCA results became clear. These are required, in order to provide comparable results and enable broader recommendations regarding sustainable biosurfactant production and use in the future. Furthermore, to implement sustainable processes on industrial scale and find suitable applications

for biosurfactants, LCA studies can be a useful tool, as they provide a holistic overview over the life cycle of a product and its environmental impacts on various levels. In this regard, this study provided an overview of key findings in existing studies and pointed out relevant research gaps. On this basis, future research can contribute to closing these gaps and leading to truly sustainable biosurfactants.

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Correction to: Environmental Impacts of Biosurfactants from a Life Cycle Perspective: A Systematic Literature Review

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The original version of the book was inadvertently published without the following corrections. The chapter has now been corrected.

Corrections:

The chapter "Environmental Impacts of Biosurfactants from a Life Cycle Perspective: A Systematic Literature Review" was mistakenly published non-open access. This has been amended with the license updated to CC BY 4.0 and the Copyright Holder changed to "The Author(s)". The book has also been updated with this change.

Page iv: The corresponding OA information has been included.

The updated original version for this chapter can be found at https://doi.org/10.1007/10_2021_194