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Biotechnological opportunities in biosurfactant production

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In the recent years, biosurfactants proved to be an interesting alternative to petrochemically derived surfactants. Two classes of biosurfactants, namely glycolipids and lipopeptides, have attracted significant commercial interest. Despite their environmental advantages and equal performance, commercialization of these molecules remains a challenge due to missing acquaintance of the applicants, higher price and lack of structural variation. The latter two issues can partially be tackled by screening for novel and better wild-type producers and optimizing the fermentation process. Yet, these traditional approaches cannot overcome all hurdles. In this review, an overview is given on how biotechnology offers opportunities for increased biosurfactant production and the creation of new types of molecules, in this way enhancing their commercial potential.

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Introduction

Surfactants are known since ancient times. The earliest evidence of soap-making dates back to the Babylonians 2800 years BC. Throughout history, more applications, molecule types and resources were discovered. In 2012, the global surfactant market generated a revenue of 27 billion dollars and is expected to rise. Most of these surfactants are obtained by chemical processes from petrochemical and oleochemical resources. During recent years, a shift towards more environmentally friendly surfactants is observed, caused by a growing awareness of both consumers and companies for the adverse effects that surfactants can have on the environment [1]. Biosurfactants, surfactants produced by micro-organisms from renewable resources, are a worthy alternative for the classic petrochemical surfactants. Because of their interesting properties like low ecotoxicity [2], easy biodegradability [3] and mild production conditions, biosurfactants have numerous potential applications in a wide variety of industrial sectors [4].

Still, some obstacles remain. Often issues like low titers combined with high production costs hamper market penetration and limited structural variation hinders integration in all application domains. To counter these problems, a lot of effort has been put into engineering the production process using either waste streams [5•], optimizing the fermentation parameters and product recovery [6•]. With the rise of biotechnical engineering, new ways for improving the production of biosurfactants became available. Both the engineering of wild-type producers as the development of heterologous production systems are viable approaches towards increased titers and enlarged molecular diversity.

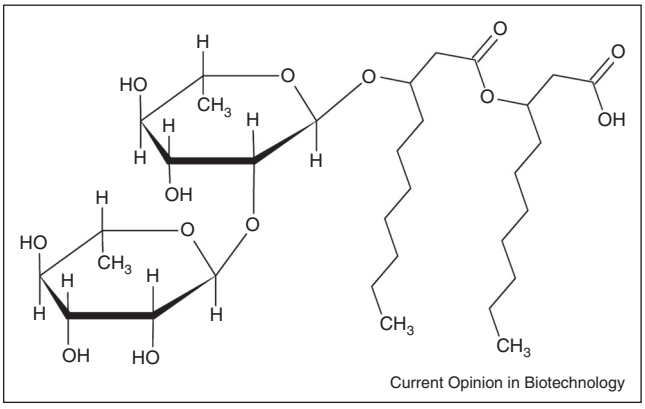
Two classes of biosurfactants are currently considered of industrial and economical relevance: glycolipids and lipopeptides, both low-molecular weight biosurfactants. The ones discussed in this review are the rhamnolipids, sophorolipids, cellobiose lipids and mannosylerythritol lipids for the glycolipids. Lipopeptides will be discussed in a more general way.

Rhamnolipids

Rhamnolipids, perhaps the most studied glycolipids to date, are composed of one or two L-rhamnose molecules coupled to a mono or dimer of β-hydroxy fatty acids [7] (Figure 1). They have interesting applications in, for example, soil remediation [8] or pest control [9]. Rhamnolipids are produced by several species from the *Pseudomonas* [10] and *Burkholderia* [11] genera with *Pseudomonas aeruginosa* being the top producer with titers over 100 g/L. Rhamnolipids are commercialized by a couple of companies like Jeneil Biotech Inc. and Rhamnolipid Companies Inc., but the high price still hampers further market penetration.

The biosynthesis of rhamnolipids depends on two pathways delivering the necessary molecules. The first one is the production of dTDP-L-rhamnose by the *rmlABCD* operon; glucose-1-phosphate is converted to the activated rhamnose that serves as a donor for the hydrophilic part of the rhamnolipids. The hydrophobic part originates from β-hydroxy fatty acids coupled to an acyl carrier protein. Two of these molecules are coupled by RhlA

Figure 1



Structure of the dirhamnolipid α-L-rhamnopyranosyl-α-L-rhanopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate. Further variation is possible in the hydrophobic part by incorporation of β-hydroxy fatty acids ranging in length from 8 to 16 carbon atoms and potential unsaturations.

to form 3-(3-hydroxyalkanoyloxy)alkanoic acids. Finally, RhlB and RhlC will link the hydrophilic and hydrophobic part to form respectively monorhamnolipids and dirhamnolipids [7].

In *P. aeruginosa*, direct and indirect factors like quorum sensing, nutritional status or stress response influence rhamnolipid production [12]. Various attempts have been made to interfere with these regulatory mechanisms. AlgR, a regulator involved in several pathways like alginate production [13], has an impact on rhamnolipid production. A recent paper proves the direct involvement of AlgR in the expression of the *rhlAB* operon by binding to the promotor [14]. Deletion of this regulator shows an increased rhamnolipid production in biofilm compared to the wild-type PAO1-strain [15]. Unfortunately, no differences could be observed when the strains were grown in liquid medium. RhlR, a regulatory protein binding to the promoter of the *rhlAB* operon, has a more subtle role. Depending on the butanoyl-homoserine lactone (C4-HSL) levels, an autoinducer that binds directly to RhlR, this regulator works either as an activator or as an inhibitor for the transcription of the operon [16]. When no C4-HSL is present, RhlR acts as an inhibitor. Mutant strains deficient in *lasR*, an essential gene for the production of C4-HSL, show no production of rhamnolipids [17]. Still, even when RhlR is present with its autoinducer C4-HSL, production is not always guaranteed [18]. Other regulators like PtxR, RsaL, RsmA, DksA and several genes encoding sigma factors like RpoN and RpoS also influence the expression levels of the *rhlAB* operon [12,19,20]. Great potential lies in uncoupling the production of rhamnolipids from this complex regulatory system. Another strategy for enhancing rhamnolipid production is the creation of a *P. aeruginosa* strain harboring the *Vitreoscilla* hemoglobin gene *vgb*. Such strains show a 7-fold increase compared to the wild-type [21]. This might be correlated to the higher oxygen uptake which enhances cell density and production.

Production of rhamnolipids results in a mixture of both monorhamnolipids and dirhamnolipids. By knocking-out *rhlC*, encoding a rhamnosyltransferase, only monorhamnolipids are produced without a big loss in production titers [22].

Engineering of species other than *P. aeruginosa* has been reported as well. Using this strategy, the opportunistic pathogenic nature of this micro-organism, which is a serious burden both on the production and application, can be circumvented. Usually, the *P. aeruginosa rhlAB* operon is expressed in a strain that is resistant towards high biosurfactant concentrations [23]. Using several *Pseudomonas putida* strains like KT2440 [23], KT2442 [24] and KCTC1067 [25], titers ranging from 0.6 g/L [24] to 7 g/L [25] were obtained. This lower production compared to *P. aeruginosa* might be explained by insufficient production of the dTDP-L-rhamnose precursor, a necessary substrate for the rhamnosyltransferases [25].

Other production hosts were evaluated as well, such as *Escherichia coli* strains W2190 [26] and BL21 [27]. When using the same strategy as described above, maximum production titers of 0.18 g/L are feasible. Again, it is suggested that the flux through the dTDP-L-rhamnose synthesis pathway is too low to guarantee higher production titers [26]. By introducing the *rhlAB* operon from *P. aeruginosa* PAO1 in *Burkholderia kururiensis* KP23, a 6-fold increase from 0.78 g/L to 5.67 g/L could be detected [28•].

Sophorolipids

Sophorolipids, another well-studied type of glycolipids, are produced by certain yeast strains like *Starmerella bombicola* [29], *Wickerhamiella domercqiae* [30] and *Candida batistae* [31]. The molecules are constituted out of a sophorose molecule (2-O-β-D-glucopyranosyl-D-glucopyranose) linked to a terminal or subterminal hydroxylated fatty acid (Figure 2). The first step in the biosynthesis of sophorolipids is the terminal or subterminal hydroxylation of a fatty acid by the cytochrome P450 monooxygenase enzyme CYP52M1. In a stepwise manner, the UDP-glucose dependent glucosyltransferases UgtA1 and UgtB1 add the two glucose units to form a non-acetylated acidic sophorolipids. These can undergo further modifications like acetylation by an specific acetyltransferase and lactonization between the free carboxylic end and the C4″ of the sophorose unit, which is governed by an extracellular lactone esterase [32,33,34]. Sophorolipids are used in several products like hard surface cleaning products from companies like Ecover and Saraya. *S. bombicola*, known for the efficient production of

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