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PRODUCTION AND APPLICATIONS OF TREHALOSE LIPID BIOSURFACTANTS

Review

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Abbreviations

SAC Surface active compounds

CMC Critical micelle concentration

TMM Trehalose mono mycolate

TDM Trehalose di mycolate

STL Succinoyl trehalose lipids

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ABSTRACT

Different types of trehalose containing glycolipids are known to be produced by several microorganisms belonging to the mycolates group such as *Mycobacterium, Rhodococcus, Arthrobacter, Nocardia,* and *Gordonia* and different structures have been elucidated particularly in *Rhodococcus* genus. Trehalolipids have gained increased interest for their potential applications in a number of areas due to their ability to lower interfacial tension and increase pseudosolubility of hydrophobic compounds. The most widespread application is in bioremediation technologies as such compounds are known to enhance bioavailability of hydrocarbons. In comparison to other microbial glycolipids, trehalolipids have generally showed contrasting results and achievements with both cases of inhibition and enhancement of biodegradation rates. One of the important aspects regarding potential use of trehalose lipids in a variety of applications is the ability to optimize their production and downstream processing. In fact, the purification of the target biological compounds by downstream processing can account for over half the production cost in many biotechnology applications. This is especially true in the case of the *Rhodococcal* glycolipids, which are often bound to cellular envelopes and are usually produced along with other surface active lipids. In this review we highlight the current knowledge of trehalolipids biosurfactant's applications and the latest strategies employed to reduce the cost of their production.

1. INTRODUCTION

1.1 Microbial surface active compounds

Biological Surface Active Compounds (SACs) are synthesized by different prokaryotic and eukaryotic organisms and are characterized by the presence of both hydrophilic and hydrophobic moieties which enables them to adsorb and alter the conditions at interfaces. According to Neu [1], biological SACs are classified into three classes: (i) biosurfactants, (ii) amphiphilic polymers and (iii) polyphilic polymers. Biosurfactants are low molecular weight SACs (e.g. glycolipids, lipopeptides) able to reduce the surface tension of water to 25-30 mN/m. The surface tension reaches its minimum value at a concentration of biosurfactant called critical micelle concentration (CMC) above which the molecules are associated, forming supermolecular structures.

Amphiphilic (e.g. lipopolysaccharides, lipoteicoic acids) and polyphilic (e.g. hydrophobic polysaccharides, emulsan) polymers are high molecular weight SACs characterized by the presence of hydrophobic groups at one end or distributed along the entire molecules, respectively. Due to their high molecular weight, often greater than 10 KDa, they are normally characterized by having CMCs higher than low molecular weight biosurfactants and they are unable to reduce the surface tension of water below 35-40 mN/m. The main property of high molecular weight SACs, is their ability to stabilize oil/water emulsions and are therefore called bioemulsifiers [2].

Glycolipids and lipopeptides are the most common low molecular weight SACs. Glycolipids are commonly mono or disaccharides acylated with long chain fatty acids or hydroxyl fatty acids. Among them, rhamnolipids, sophorolipids and trehalolipids are the best-studied structural subclasses. Rhamnolipids are produced by different *Pseudomonas* species, sophorolipids are synthesized by different species of the yeast *Candida* (formerly *Torulopsis*) and trehalolipids are found in *Rhodococcus* and other actinomycetes [3]. Lipopeptides are low molecular weight SACs and the most extensively lipopeptides are produced by several *Bacillus* species; particularly *Bacillus subtilis* that produces surfactin, a cyclic lipopeptide considered the most active biosurfactant discovered so far [4].

Different *Acinetobacter* species produce well-known high molecular weight bioemulsifiers such as emulsan, an emulsifier produced by the *Acinetobacter lwoffii* strain RAG-1 (formerly *Acinetobacter calcoaceticus*). Emulsan is a complex mixture of an anionic heteropolysaccharide and proteins. It represents a polyphilic structure with fatty acids attached, over the entire molecule, to the polysaccharide backbone [5].

Van Hamme *et al.* [6] recently reviewed the physiological roles of microbial SACs. Motility (gliding, swarming, de-adhesion from surfaces), cell-cell interactions (biofilm formation,

maintenance and maturation, quorum sensing, amensalism, pathogenicity), cellular differentiation, substrate accession and resistance to toxic compounds are some of the main roles attributed to microbial SACs. However, the most widespread role of microbial SACs is believed to be the interaction between microbes and unsoluble substrates such as hydrocarbons.

2. TREHALOSE LIPIDS

2.1 Chemical structures and roles

Trehalose is a non-reducing disaccharide in which the two glucose units are linked in an α,α-1,1-glycosidic linkage. It is the basic component of the cell wall glycolipids in *Mycobacteria* and *Corynebacteria*. The most reported trehalose lipid is trehalose 6,6'-dimycolate, which is a α-branched-chain mycolic acid esterified to the C6 position of each glucose. Different trehalose containing glycolipids are known to be produced by several other microorganisms belonging to mycolates group such as *Arthrobacter, Nocardia, Rhodococcus* and *Gordonia*. Particularly in *Rhodococcus* genus, several types of trehalose lipids have been elucidated (Figure 1) [7]. These glycolipids vary in the number and overall chain length (C20–C90) of the esterified fatty acids.

Characterisation of the organic extract of *Rhodococcus erythropolis* DSM43215 by Kretschmer *et al.* [3] revealed the presence of trehalose-6-monocorynomycolates, trehalose-6,6'-diacylates (e.g. 3-oxo-2-alkyl alkanoic acid) and trehalose-6-acylates (eg. 3-oxo-2-alkyl alkanoic acid). Trehalose lipids were subsequently isolated from *Rhodococcus erythropolis* by Ristau & Wagner [8]. The glycolipid synthesised by *Rhodococcus* strain H13-A is a nonionic trehalose lipid, consisting of one major and 10 minor components [9]. Kurane *et al.* [10] reported flocculating properties caused by glycolipids of *R. erythropolis* S-1; the carbohydrate is acylated with C10–C22 saturated and unsaturated fatty acids, C35–C40 mycolic acids, hexanedioic, dodecanedioic acids, 10-methyl hexadecanoic and 10-methyl octadecanoic acids. Several studies have resulted in the discovery of novel types of trehalolipids including: mono-, di- and tri-corynomycolates [11-13], mono-, di-, tetra-, hexa and octa-acylated derivatives of trehalose [9, 11], trehalose tetraesters [8, 14-17] and succinoyl trehalose lipids [18, 19].

Most of the trehalose lipids synthesized by *Rhodococcus* and related genera are bound to cell envelope and are produced mainly when the microorganisms are grown on hydrocarbons. These characteristics have significant negative consequences on the level of production and their recovery for industrial applications.

In alkanotrophic mycolates trehalose lipids are thought to have a key role in accessing hydrocarbon substrates. Several strategies are used by bacteria to overcome the low solubility of hydrocarbons and enhance their transport [20, 21]. The ability of different microorganisms to access hydrocarbons

depends on their cell surface hydrophobicity. Cells with high hydrophobicity allow microorganisms to directly contact oil drops and solid hydrocarbons while low cell hydrophobicity permits the adhesion of microbial cells to the micelles or emulsified oils, formed due to the presence of extracellular biosurfactants or bioemulsifiers [22, 23]. In mycolates, the mycolic acid layer confers high hydrophobicity to the cell surface. For this reason, the major hydrocarbon accession mode is likely to be direct contact of hydrophobic cells with large oil drops [20]. Furthermore, microorganisms can increase or decrease their surface hydrophobicity by locating the hydrophobic moieties of cell-bound biosurfactants outwardly or inwardly, respectively [4]. Recently, Franzetti *et al.* [24] suggested that this regulation can lead to changes in the substrate access mode during the different growth stages on hydrocarbons. They observed that the cells of *Gordonia* sp. BS29 are hydrophobic during early exponential phase of growth on n-hexadecane and access to large oil drops is by direct contact. During the late exponential phase, changes occur so that the cell surface becomes hydrophilic. Cell bound glycolipids accumulate during growth reducing the surface hydrophobicity, exposing their hydrophilic moieties toward the water phase, thus masking the highly hydrophobic character of the mycolic acid layer. At the same time, *Gordonia* sp. BS29 releases extracellular bioemulsifier allowing hydrophilic cells to attach to the hydrophilic outer layer of the emulsified oil droplets.

2.2 Biosynthetic pathways

While the formation of mycolic residues is believed to be a Claisen-condensation, the key reaction for synthesis of the final resulting sugar residue, trehalose-6-phosphate, is catalysed by a trehalose-6-phosphate synthetase (TPS) which links two D-glycopyranosyl units at C1 and C1'. UDP-glucose and glucose-6-phosphate act as the immediate precursors [25]. In alkanotrophic rhodococci, TPS is induced by n-alkanes [7]. The further reactions involved in the synthesis of trehalose lipids have been clearly elucidated for trehalose dimycolates (TDM) in *M. tubercolosis* in which the production occurs in the final stages of the synthesis of the cell wall [26]. In this phase newly synthesized mycolic acids are transported and attached to the peptidoglycan-arabinogalactan complex of the cell wall, followed by the formation of TDM occurs by four different reactions (Fig.2). The synthesis proceeds through the transfer of the mycolyl group to D-mannopyranosyl-1-phosphoheptaprenol by a proposed cytoplasmic mycolyltransferase I to form 6-*O*-mycolyl-β-D-mannopyranosyl-1-phosphoheptaprenol (Myc-PL) (Fig. 2, reaction 1). The mycolyl group is then transferred to trehalose 6-phosphate by a membrane-associated mycolyltransferase II (reaction 2) to form Trehalose Mono Mycolate (TMM)-phosphate and, after dephosphorilation, results in formation of TMM. TMM is transported outside the cell by a ABC transporter (reaction 3). A rapid and efficient

transfer of TMM from the inside to the outside of the cell is necessary for the synthesis of cell wall arabinogalactan-mycolate and TDM. By the action of the extracellular mycolyltransferase called Ag85/Fbp/PS1, the final products of the cell wall arabinogalactan-mycolate and TDM are formed from TMM (reactions 4).

3. PRODUCTION AND ANALYSIS OF TREHALOSE LIPIDS

3.1 Carbon substrates and production

Key points for the microbial production of trehalose lipids are the substrate used for production and the ability to release the glycolipids. In 1998, Lang and Philp demonstrated that the alkanotrophic ability of the strains and the production of cell-bound biosurfactants are specific features of trehalose lipid production in *Rhodococcus* genus. Subsequently, several papers have been published confirming this behaviour [17] [27-29]. However, in recent years several studies reported production of biosurfactants both extracellularly and on soluble substrates. *R. erythropolis* ATCC 4277 was able to produce extracellular glycolipids grown on a medium containing glycerol as sole carbon source and released all the trehalose lipid into the medium, while the production was partially cell-bound when cells were grown on n-hexadecane [30]. Trehalose and other lipids were detected among the surface active compounds produced by *R. erythropolis* EK-1 grown on various soluble and unsoluble carbon sources [12]. *Rhodococcus sp*. SD-74 produces extracellular succinoyl trehalose lipids when cultivated on n-hexadecane [19]. However, when n-hexadecane was supplied as the sole carbon source, two types of biosurfactants (free fatty acids and trehalose lipids) were detected in the supernatant of the bacterial culture [31].

Furthermore, some authors recently described production of both cell-bound biosurfactants and extracellular emulsifiers in *Rhodococcus* and related genera. The cell bound biosurfactants are able to reduce the surface tension and seem to be produced only on hydrocarbon substrates while the extracellular bioemulsifiers are produced also on soluble substrates [12, 24, 32, 33]. Table 1 displays some examples of *Rhodococcus* isolates with the indications of the chemical structures produced, the carbon substrates used and the position of the products.

3.2 Optimisation of production

In a recent review [34] Mukherjee *et al.* stated that microbial surfactants commercialization has not been accomplished so far, despite their characteristics of lower toxicity, higher biodegradability, better foaming properties than synthetic counterparts and while also having stable activity at extreme pH, salinity and temperature. While these highly favourable properties have been known and discussed previously [35], their commercial exploitation on a large scale has yet to occur .

Commercial success and efficiency of the entire biotechnological process is a key point for microbial surfactants, as is for most biotechnological products. This is especially true for trehalose lipids which are often found bound to cell surface, thus increasing down-stream costs and reducing production yield. Different strategies have been adopted in order to make the process cost-competitive including: (1) use of cheap and waste substrates (2) development of efficient bioprocesses, including optimization of fermentative condition and recovery process, (3) development of overproducing strains. However, biosurfactant research related to production enhancement and economics has been confined, mostly, to a few genera of microorganisms, such as *Bacillus, Pseudomonas and Candida*, while a large group of biosurfactant producers belonging to the genera *Rhodococcus, Gordonia,* and *Acinetobacter* have not yet been exploited [34].

The ability to increase the production and the recovery of these glycolipids from the cell envelop is necessary before exploitation can occur [7, 35-37]. Several papers have studied the cultural factors affecting the production and the use of alternative low-cost substrates. One such study has shown that at high concentration of phosphate buffer and neutral pH conditions the production of succinoyl trehalose lipids in *R. erythropolis* SD-74 was optimised [18]. The nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodococcus* bacterium isolated from Kuwaiti soil have been determined [38]. While, Espuny *et al.* [39] reported a growth-dependent production of biosurfactant by *Rhodococcus* sp. and determined n-tridecane to be the best carbon source for the trehalose lipid production for this strain, resulting in an increase from 0.5 g/L to 3 g/L of glycolipid.

More recently, experimental design techniques have been applied for the optimisation of biosurfactant production, resulting in increased production yields. Using a step-wise approach, Franzetti *et al.* [40] increased the production of cell-bound glycolipids of *Gordonia* sp. BS29 by 5-fold. The production of biosurfactant from *Rhodococcus* spp. MTCC 2574 on n-hexadecane was effectively enhanced by surface response methodology. The yield of biosurfactant increased from 3.2 g/L to 10.9 g/L [28]. Another approach aimed at reducing the cost of production is the use of low-cost substrate. *R. erythropolis* 16 LM.USTHB converted residual sunflower frying oil, a cheap renewable substrate into extracellular glycolipids lowering the surface tension of the crude broth down to 31.9 mN/m [33]. During a screening study of biosurfactant producers on renewable low-cost substrates, Ruggeri *et al.* [41] isolated *Rhodococcus* sp. BS32 able to produce extracellular biosurfactants growing on rapeseed oil.

Only one study detailing the use of recombinant strains for trehalose lipids production has been reported. A recombinant *Gordonia amarae* was developed by insertion, stable maintenance and expression of the *Vitreoscilla* hemoglobin gene (*vgb*), resulting in enhanced production of the trehalose lipid biosurfactants in the engineered strain [42].

3.3. Downstream processes

The most commonly used solvent system for efficient extraction is chloroform:methanol (2:1) [43], while methyl tert butyl ether (MTBE) [29] and more recently a mixture of ethyl acetate:methanol (8:1) has been shown to be a suitable solvent for extraction [44]. An approximate determination of trehalose lipid content in a culture medium or an extract can be carried out in a similar manner to other glycolipids using the anthrone method [45]. This colorimetric assay works by reacting anthrone with the sugar part of the trehalose using assay to form a coloured complex, which can be quantified using a spectrophotometer. Phenol-sulfuric acid method has also been used for quantification [46].

Purification of trehalose lipids is generally carried out using either Thin Layer Chromatography (TLC) or column chromatorgraphy. TLC has been extensively used to detect trehalose lipids in an extract while also providing information about the structural composition. Several solvent systems have been reported but the most extensively used system is chloroform:methanol:water (65:15:2 or 65:25:4), which allows for purification of milligram quantities [3]. Using p-anisaldehyde trehalose lipids will appear green, with trehalose monomycolates appearing near the point of origin with trehalose dimycolates slightly above. Other spots are likely to be detected corresponding to other lipid components of the trehalose lipid extract.

Large scale purification using column chromatography is a laborious undertaking as these molecules are generally produced at low concentrations and thus represent a minor component of the crude extract sample. The presence of different structural types of trehalolipids and a large number of other lipids type material along with excess *n*-alkane used as substrate in the production process complicates the purification process further. Consequently a preliminary column chromatography step has been suggested to remove hydrocarbon before a subsequent column chromatography for the purification of trehalose lipids is carried out using chloroform:methanol mixtures [3]. While others have carried out a one step column purification, with the difference in each method mainly due to different increments of chloroform:methanol mixtures [18, 47, 48].

3.4. Structural characterization

Structural characterization of purified trehalose lipids can also be carried out using numerous techniques working either on the intact molecule or by breaking down the structure into carbohydrate and fatty acid components. Mass spectrometry provides the best method for characterisation of trehalose lipids.

Gas Chromatograpy Mass spectrometry (GC-MS) is used extensively for the characterisation of the fatty acid components or the carbohydrate portion of trehalose lipids. After alkaline hydrolysis of the glycolipid mixture, conversion of the lipid portion to fatty acid methyl esters enables the use of GC or GC-MS to determine the structure. This technique is reported in the majority of paper concerned with trehalose lipids [3, 29, 49]. Characterisation of the fatty acid profile provides essential information needed to identify the structure. Analysis of the trehalose component using GC and GC-MS, after conversion to trimethylsiliyl derivatives, provides information on the ester linkages of the fatty acids to trehalose [43, 49].

Analysis of the intact trehalose lipid is extremely useful for determination of the molecular weight of the glycolipids present. Since each type of trehalose lipid is present as a mixture due to different fatty acid compositions, intact mass spectrometry can be used to identify all individual structures. The molecular weight along with GC-MS analysis of the fatty acids present is generally enough for total characterization. Fast Atom Bombardment Mass Spectrometry (FAB-MS) [48, 50] and more recently Matrix Assisted Laser Desorption Ionisation (MALDI) [51] have been demonstrated for characterization of the trehalose lipid structures. In recent times the use of ElectroSpray Ionisation Mass spectrometry (ESI-MS) has also been reported for analysis [47]. However, this study used positive ionisation mode which can become very complex due to the presence of both protonated ions along with sodium adducts. Therefore, ESI-MS in negative ion mode would be a better alternative and this was demonstrated in a recent publication using HPLC-MS [44]. Nuclear Magnetic Resonance (NMR) can also be used for characterisation of trehalose lipids either intact or after hydrolysis. NMR analysis of the intact is relatively difficult to interpret, therefore analysis of the trehalose portion is more preferred affording information that helps to characterise the position of where the fatty acids attach to the carbohydrate structure [18, 39, 47].

4. POTENTIAL APPLICATION OF TREHALOSE LIPIDS

The use of biosurfactants has been proposed for a number of different commercial applications. At present, the main applications are found in the hydrocarbon bioremediation and oil and petroleum industry, in particular for microbial enhanced oil recovery (MEOR) and oil storage tank cleaning. Another emerging field of application is the biomedical/healthcare industry, since some biosurfactants have already been demonstrated to be suitable alternatives to synthetic products as antimicrobial and therapeutic agents. Biosurfactants also have potential applications as additives for agricultural use, food industry, mining and manufacturing processes, pulp and paper industries, and as detergents or cosmetics [36]. The use of trehalose lipids has been reported in the environmental field, as additives which could potentially enhance solubility of hydrophobic compounds and

stimulate biodegradation of hydrocarbons in contaminated soils, while also showing promise for enhanced oil recovery. Furthermore, their use has been proposed in therapeutic applications, due to their biological activity.

4.1 Environmental applications

In many cases, environmental contamination caused by industrial activities is due to hydrophobic organic compounds. Such compounds generally pose problems for remediation, as they get easily bound to soil particles which renders them less soluble and bioavailable to microorganisms that can potentially degrade them. The application of biosurfactants in the remediation field is therefore aimed at enhancing solubility of organic compounds, either for a soil washing treatment, or to stimulate *in situ* biodegradation. In particular, the application of trehalose lipids generally showed good results in solubilisation and biodegradation experiments with different hydrophobic organic compounds. Oberbremer *et al.* [52] added different glycolipids to a model system containing 10% soil and a hydrocarbon mixture; they observed decreased adaptation times of the inoculum and an increase in the extent of hydrocarbon degradation and final biomass concentration. In one study of field treatment, the addition of *Rhodococcus ruber* strain IEGM AC219 and surfactant complexes from various *Rhodococcus* strains to windrows of crude oil contaminated agricultural soil slightly enhanced hydrocarbon degradation over a three-month period. Degradation of hydrocarbons was further enhanced when biopile systems were set with increased ventilation, nutrient addition and bulking with straw [37, 53]. The same biosurfactant complexes were also used for *in situ* stimulation of autochthonous crude oil degrading bacteria in oil-contaminated soils. In this case, the introduction of the biosurfactant resulted in increased oil degradation and crude oil degrading bacteria population [37]. *Mycobacterium flavescens* strain EX-91 was used for the development of a commercial product, named Ekoil, which was tested in the decontamination of an oil-polluted water body, and also proved effective in the treatment of the engine oil-contaminated wastewater of a nuclear power station [54].

Van Dike *et al.* [55] reported that the biosurfactants produced by *Rhodococcus erythropolis* performed poorly in desorption tests of hydrophobic compounds from soil, but such results were attributed to the use of cell-free culture media, when it is known that the majority of *Rhodococcal* surfactants are cell-bound. In comparison Park *et al.* [56] reported that the biosurfactant produced by *Nocardia erythropolis* had a partitioning capacity for *p*-xylene of an order magnitude greater than that of sodium dodecyl sulphate. The addition of trehalose lipids from *R. erythropolis* could increase the apparent solubility of phenanthrene up to more than 30-fold the reported aqueous solubility. Furthermore, the addition of the same trehalose lipids significantly enhanced the rate and

the extent of phenanthrene mineralisation by the phenanthrene degrading isolate P5-2 in liquid cultures and in spiked soil. However, it only increased the rate but not the extent of mineralisation in slurry phase [57]. A biosurfactant produced by *Rhodococcus* strain H13-A was more effective than the synthetic surfactant Tween 80 in the enhancement of the aqueous concentrations of several Polycyclic Aromatic Hydrocarbons (PAHs) from crude oil. The enhanced PAH concentrations ranged from 2.2 times to more than 35 times for the biosurfactant treatment compared to the synthetic surfactant treatment [58]. In a recent study, Peng *et al.* [31] reported an increase of 4.4, 1.3 and 23.3-fold, respectively, in apparent solubility of dibenzothiophene, naphthalene and phenanthrene in water, when an extract of biosurfactants from *R. erythropolis* strain 3C-9 was added. In such a case, the more hydrophobic the substrate, a more enhanced solubility was observed. In contrast, Franzetti *et al.* (2009) [59] reported that biosurfactants produced by *Gordonia* sp. strain BS29, while effective in enhancing crude oil and PAH removal by soil washing, were generally not able to increase the rate or extent of their biodegradation.

One key point in the application of biosurfactants to environmental remediation is their specificity, due to the fact that different microbial strains produce different molecules. In some studies, it was demonstrated that the correct biosurfactants and surfactant-producing strains should be used to obtain a better performance in the remediation treatments. For example, degradation of *n*-hexadecane was stimulated by rhamnolipid in *Pseudomonas aeruginosa*, but not in *Rhodococcus* strains, and the same *P. aeruginosa* was stimulated only by its own rhamnolipid, thus demonstrating that the effects of biosurfactants may be specific [60]. Nevertheless in contrast to this study, biosurfactants from *R. erythropolis* strain 3C-9 significantly increased the degradation rate of *n*-hexadecane by two phylogenetically distant strains, *Alcanivorax dieselolei* and *Psychrobacter celer,* in flask tests [31], demonstrating the conflicting results within this field. Trehalose lipids have been generally used in bioremediation of contaminated soils; at present, there is only one proposed application for the treatment of wastewater. Trehalose corynomycolates produced by *Rhodococcus erythropolis* S-1 was demonstrated to be important in the flocculating activity of the strain [10]. The flocculant in the culture broth was hypothesised to form micelles composed of proteins and the trehalose lipids, suggesting that such activity could be useful in the removal of suspended solids from wastewater.

It is generally believed that biosurfactants are more environmentally friendly alternative to synthetic surfactants because of their lower toxicity and higher biodegradability. However, toxicity of microbial produced surfactants should always be assessed, especially when an *in situ* application is planned. Munstermann *et al.* [61] verified that trehalose tetraester from *Rhodococcus erythropolis* was less toxic to *Vibrio fischeri* (acute Microtox® toxicity test) than trehalose dicorynomycolate

from the same strain and rhamnolipids from *Pseudomonas aeruginosa*, and that it was also much less toxic than a number of synthetic surfactants and bioremediation formulations. Ivshina *et al.* [53] found that a *R. ruber* glycolipid complex was even less toxic than all of the (bio)surfactants cited by Munstermann as having an IC_{50} more than 10 times higher than the CMC. They showed that their products has a toxicity 100-1000 times less than synthetic surfactants, 2-10 times less than trehalose lipids from *R. erythropolis* and 13 less toxic than rhamnolipids form *Pseudomonas aeruginosa*. Furthermore, glycolipids produced by *Rhodococcus* sp. strain 413A exhibited 50% less toxicity than Tween 80 in naphthalene solubilisation tests [62]. Another study using trehalose lipids from *R. erythropolis* did not show any toxic effect on [^{14}C] glucose mineralisation in liquid phase by a phenanthrene degrading strain P5-2 [57].

At present, the main obstacle to a full-scale application of biosurfactants in bioremediation technologies is the high cost for their production, compared with the amounts required even for a single treatment. A possible solution is the preferential performance of an *in situ* treatment, when applicable, which encourages the production of biosurfactants *in situ* rather than costly bioreactors processes. The best strategy in this case would be the identification and the selective stimulation of autochthonous biosurfactant-producing bacteria [63]. Using this methodology, particular attention should be paid to the *in situ* conditions. For example, the production of biosurfactants has often been associated with nitrogen limitations, so that over-fertilization, which is a common practice for *in situ* remediation, would have a negative effect. *Rhodococci* may be good candidates for an *in situ* stimulation, as they were often found to be the dominant component in microbial communities present at oil-polluted sites [37]. If biosurfactant producers are not present in the site to be remediated, they can be nevertheless introduced, assuming that the introduced microorganisms would survive over time. Christofi and Ivshina [37] studied the dynamics of rhodococcal population in soil after inoculation of *R. erythropolis* and *R. ruber* into an oil-contaminated soil. While *R. erythropolis* showed a sharp increase during the first month, the number of *R. ruber* remained almost constant. However, the simultaneous introduction of the two strains resulted in a 75.5% decrease in the oil content in three months. When the site conditions make it necessary to turn to a soil washing rather than an *in situ* treatment, the recycling of biosurfactants in the washing solution should be carried out, in order to minimise the costs of the whole operation. However, although a reasonable quantity of biosurfactant is required for a remediation treatment, there is no strict need of product purity, allowing cell-free culture broths to be directly employed without undergoing complex downstream processing or purifications [37]. In other cost cutting measures for such remediation's it was synthetic mycolic acid surfactant that is synthesised by a simple and cost-

effective pathway has been used as an additive for the enhancement of diesel oil biodegradation [64]. Such additive resulted in a greater efficiency in stimulating hydrocarbon degradation.

4.2 Industrial applications

Poor oil recovery from oil-wells may be due to either low permeability of the rocks forming the reservoir or alternatively the high viscosity of the crude oil, which inhibits its mobility. Microbial enhanced oil recovery exploits the ability of autochthonous or injected microorganisms to synthesise products which may improve oil recovery from the oil reservoirs [65]. One of the first documented applications for biosurfactants concerned the use in enhanced oil recovery, due to their ability to reduce the oil/water interfacial tension. Three different strategies can be employed in enhanced oil recovery; biosurfactant production in reactor cultures and subsequent addition to the oil reservoir; biosurfactant production by injected microorganisms; and finally injection of nutrients into the reservoir to stimulate *in situ* biosurfactant production by autochthonous bacteria [65]. At present, the first strategy appears to be the most studied, even if production costs are definitely higher than for the later two. Finnerty and Singer [66] demonstrated that the trehalose glycolipid produced by *Rhodococcus* strain H13-A improved the displacement of crude oil from rock cores by 20%, while oil recovery increases of around 30% from sandstones have been reported by using trehalose lipids produced by *Nocardia rhodochrus* [43]. Recently, biosurfactants produced by *Rhodococcus erythropolis* and *R. ruber* were used to extract hydrocarbons from oil shale; the maximum recovery was obtained with biosurfactant concentrations of 8 g/L and 4 g/L for the two strains, respectively [67]. However, oil recovery proved less effective when a high percentage of asphaltenes and resin compounds were present. This result confirmed previous studies by Ivshina *et al.* [53], who demonstrated that crude biosurfactant complexes produced by *Rhodococcus* strains were effective in enhancing oil removal from sands and oil shale cuttings, even if at variable extent, but the process was less successful for oils containing increased asphaltene content. The composition of crude oil recovered from a contaminated soil matrix by a *R. ruber* biosurfactant was altered, resulting in a 3.6-fold increase in the fraction of aromatic compounds and a 5-fold decrease in the asphaltene fraction when compared to the initial oil composition [68]. On this basis, the authors suggested that *R. ruber* biosurfactant is able to remove a hydrocarbon mixture with a composition that would be more easily biodegradable by microorganisms than the original crude oil, thus proposing possible applications for *in situ* remediation treatments. An alternative to reduce the intervention costs of microbial enhanced oil recovery would be an *in situ* stimulation of the autochthonous microflora. Culture broths of a *R. ruber* strain isolated from an oil field in China proved effective for the release of oil from white sand, while several other strains originating from

the same site showed reduced surface tension in the cultivation media and production of biosurfactants [32].

The dual hydrophobic/hydrophilic nature of biosurfactants can also help microorganisms to displace other emulsifier compounds from oil/water interfaces. Such de-emulsifying property may be used to break emulsions which form at various steps of oil extraction and processing, thus allowing a better recovery of the product. Several microorganisms are known to display de-emulsifying properties; among them, various strains of *Nocardia* and *Rhodococcus*, whose properties remained unaltered even after autoclaving [69].

4.3 Biomedical applications

The use of biosurfactants in medical applications has been proposed, due to several biological properties such as antimicrobial, antiviral, anti-adhesive, anticancer or immunomodulating properties. Furthermore, biosurfactants are generally considered safer than synthetic pharmaceuticals, due to their biological origin [70]. To date there have been very few studies carried out to confirm their lack of toxicity. For example, Marquès *et al.* [44] assayed potential skin irritation of trehalose lipids produced by *Rhodococcus erythropolis* 51T7 with mouse fibroblast and human keratinocyte lines. Their results indicated that the biosurfactant is less irritating than sodium dodecyl sulphate, and could be therefore used in cosmetic preparations. Isoda *et al.* [71] investigated the biological activities of several glycolipids, including the two succinoyl trehalose lipids STL-1 and STL-3, and found that they induced cell differentiation into monocytes instead of cell proliferation in the human promyelocytic leukaemia cell line HL60. To elucidate biological interactions at the basis of such activity, four analogs of STL-3 were also evaluated for their ability to inhibit growth and to induce differentiation in the same cell line [72]. It was found that the biological effects of STL-3 and its analogs were dependent on the structure of the hydrophobic moiety of STL-3.

Trehalose-6,6'-dimycolate (TDM), or cord factor, has been extensively studied from a medical point of view due to the fact that it plays a central role in pathogenesis during infection. TDM also showed a number of different biological activities, such as antitumor activity [73, 74]; augmentation effect of nonspecific immunity to microbial infection [75]; immunomodulating functions, i.e. granuloma-forming activity [76, 77]; priming of murine macrophages to produce nitric oxide [78, 79]; induction of the production of cytokines and enhancement of angiogenic activity in mice [80]. Despite the promising pharmaceutical applications, the use of *Mycobacterial* TDM is limited by the relatively high toxicity of the molecule and the potential pathogenicity of producer strains. TDM produced by *Rhodococcus* sp. 4306 was demonstrated to exhibit lower toxicity, both *in vivo* and *in*

vitro, than *Mycobacteria* TDM. This is thought to be due to the presence of shorter mycolic acids on the *Rhodococcus* derived TDM (C_{34} to C_{38}) compared to C_{74} to C_{86} for the *Mycobacteria*l TDM [50, 80]. While the complex synthesised by *R. ruber* showed no toxicity or effect on proliferative activity of peripheral blood leukocytes [81]. These results clearly indicate that *Rhodococcal* TDM may have some pharmacological potential uses.

Trehalose lipids were also reported to have antiviral and antimicrobial properties. TDM conferred to mice higher resistance to intranasal infection by influenza virus [82]. It was demonstrated that the biosurfactant induced proliferation of T-lymphocytes bearing gamma/delta T-cell receptors (γδ T-cells), associated with the maintenance of acquired resistance to the infection [83]. Furthermore, the trehalose lipids produced by *Tsukamurella* sp. strain DSM 44370 together with trisaccharide and tetrasaccharide lipids showed some activity against gram-positive bacteria, although the pathogenic strain *Staphylococcus aureus* was not affected by them. Gram-negative bacteria were either slightly or not inhibited at all [84]. Recently, the effect of trehalose lipids from *Rhodococcus* sp. strain 51T7 on the most important membrane phospholipids was investigated. This study was carried out in order to better elucidate the molecular interactions between the biosurfactant and the lipidic component of the membrane [27, 85]. It was observed that trehalose lipid increased the fluidity of both phosphatidylethanolamine and phosphatidylserine membranes and formed domains in the fluid state, but it did not modify the macroscopic bilayer organization.

As for other applications, the most important factor limiting the use of biosurfactants as an alternative to synthetic compounds is the high cost of production and downstream processing. However, in pharmaceutical and biomedical sectors, it could be compensated for by the small amounts of product required. In fact, it has been elucidated that biosurfactants used as pharmaceutical agents are needed only in very low concentrations [86].

5. CONCLUSIONS AND PERSPECTIVES

In the past thirty years several different structural trehalolipids have been discovered and numerous producing strains have been isolated and characterised. Microbial trehalose lipids showed many interesting potential applications in different fields. In the future, our increasing ability to analyze the microbial diversity in natural environments is expected to expand our knowledge on microbial trehalolipids leading to the discovery of new chemical structures and producing strains. However, the commercial success of microbial trehalolipids is currently scarce mainly due to the high cost of production, mainly due to the presence of the molecules bound to the cellular envelop. In bioremediation, trehalolipids share with other biosurfactants, a lack of knowledge about the mechanisms of interactions among hydrocarbons, surfactants and cells which limits their extensive

application. In pharmaceutical field, in which low amount of high value product is required, the research seems to be at its infancy even if it is expected to provide a new venture for industrial investments [34].

If the research on microbial trehalolipids is to succeed in overcoming these drawbacks it will meet the expected market demands of efficient, affordable and environmental friendly surfactants.

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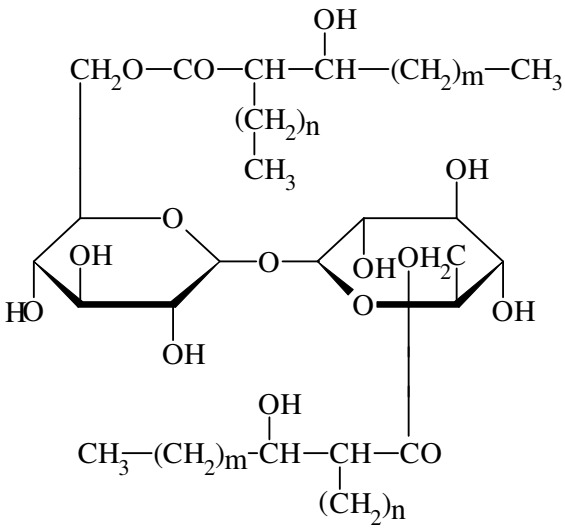
7. TABLES

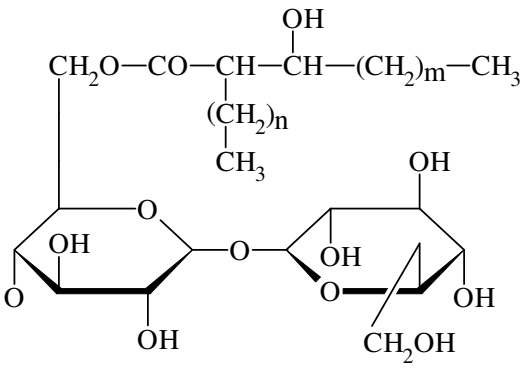
Strain	Product	Substrates	Notes	Ref.
Rhodococcus erythropolis	trehalose dicorynomycolates	*n*-alkanes	cell-bound	3
Rhodococcus erythropolis DSM 43215	trehalose-dicorynomycolates trehalose-monocorynomycolates	C_{14}- C_{15} n-alkanes or kerosene	extracellular (70%)	8
Rhodococcus H13-A	glycolipids	n-alkanes and fatty alcohol	cell-bound and extracellular	9
Rhodococcus erythropolis S1	glucose monomycolate, trehalose monomycolate and trehalose dimycolates	trehalose monomycolate, glucose monomycolate, and trehalose dimycolate	cell-bound	10
Rhodococcus ruber	trehalose dicorynomycolates	hydrocarbons	cell-bound	11
Rhodococcus erythropolis EK-1	trehalose monocorynomycolate trehalose dicorynomycolate	ethanol	extracellular	12
Rhodococcus erythropolis	trehalose-2,2',3,4-tetraester	n-alkanes	cell-bound	14
Rhodococcus erythropolis MS11	trehalose tetraester esterified with succinic acids and decanoic acid	n-hexadecane	extracellular	16
Rhodococcus wratislaviensis BN38	trehalose tetraester	n-hexadecane.	cell-bound	17
Rhodococcus erythropolis	succinoyl trehalose lipids	n-hexadecane	extracellular	18
Rhodococcus SD-74	succinoyl trehalose lipids	n-hexadecane	extracellular	19
R. erythropolis ATCC 4277	biosurfactant	glycerol	cell-bound	30
Rhodococcus erythropolis 3C-9	trehalose lipids	n-hexadecane	cell-bound	31

Table 1. The main glycolipid producing *Rhodococcus* species, their main trehalose lipid produced along with the carbon substrate used and their cellular deposition.

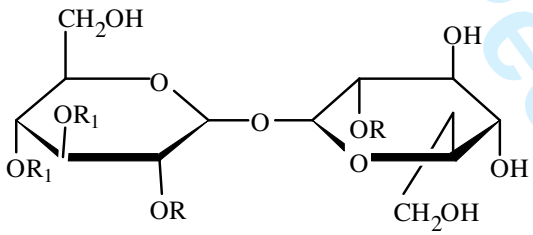
FIGURES



m+n =27 TO 31
Trehalose dimycolates



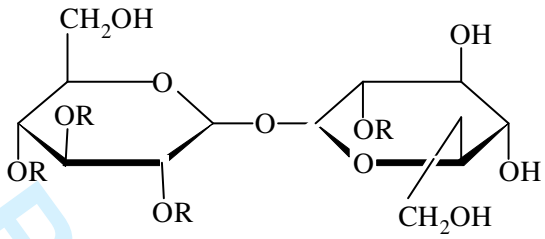
m+n =27 TO 31
Trehalose monomycolates



R = $OC(CH_2)_2COOH$

$R_1 = OC(CH_2)_mCH_3$
m= 5-9

Succinoyl trehalose lipid



R= either $OC(CH_2)_mCH_3$ + $OC(CH_2)_2CH_3$
or $OC(CH_2)_mCH_3$
m= 5-9

Trehalose tetraesters

Fig. 1. The chemical structure of the main trehalose lipids along with the most commonly reported side chains.

	Reaction	Site of action
1	Mycolyl-S-Pks13 + Man – P-heptaprenol → Myc-PL	**Inside the cell**
2	Myc-PL + Treh 6-P → TMM	
3	TMM Inside + ATP → TMM Outside + ADP+ Pi	**Transfer outside the cell**
4	TMM + TMM → TDM + Treh	**Outside the cell**

Pks13: polyketide synthase 13

Man – P-heptaprenol : D-mannopyranosyl-1-phosphoheptaprenol

Myc-PL: 6-*O*-mycolyl-β-D-mannopyranosyl-1-phosphoheptaprenol

Treh 6-P: trehalose 6-phosphate

TMM: trehalose mono mycolate

TDM: trehalose dimycolate

Fig. 2. Biosynthetic pathway of trehalose dimycolates modified from Takayama et al. [26].