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28 Isolation and Analysis of Low Molecular Weight Microbial Glycolipids

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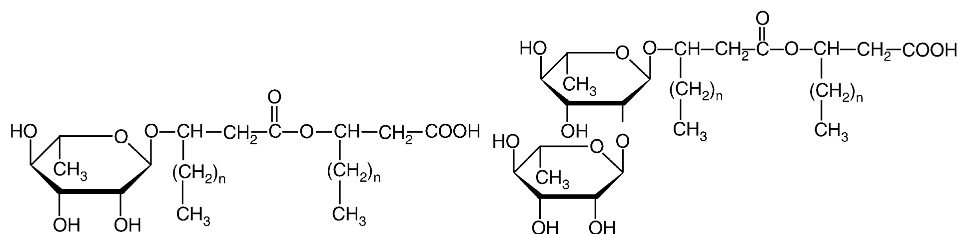
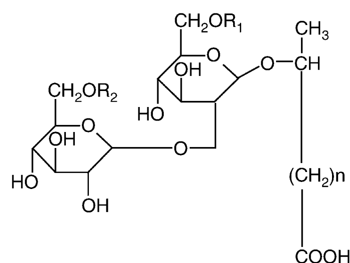
Abstract: Microbial glycolipids consist of four major groups, rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids. Extensive research has been carried out on rhamnolipids and sophorolipids, with slightly less research to date carried out on trehalose lipids and mannosylerythritol lipids. When studying these microbial glycolipids, the ability to isolate, purify and characterize the structures being produced is extremely important. This structural information provides insight into the different conditions, such as carbon sources, etc. that effect production of glycolipids. The information from analysis allows the optimization of production yields and assembly of glycolipids with different structural characteristic. Therefore, the ability to drive production in a certain direction allow the microbiologist to produce different types of glycolipids depending on the biological activity required, such as surface tension, is possible. The experimental techniques used to isolate, purify and analysis glycolipids is extremely varied, such as colorimetric assays that give rough indication of production yields, ranging to complex mass spectral techniques. Mass spectrometry provides essential information that results in the identification and quantification of individual glycolipid structures, including isomers. However, mass spectrometry requires extremely purified glycolipids for best result, which can be carried out using various chromatographic techniques. This paper therefore details information and methods that would be required to analysis glycolipids. Since there are numerous methods available, only the most commonly reported techniques are presented in this paper.

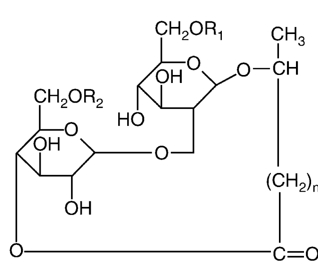
1 Introduction

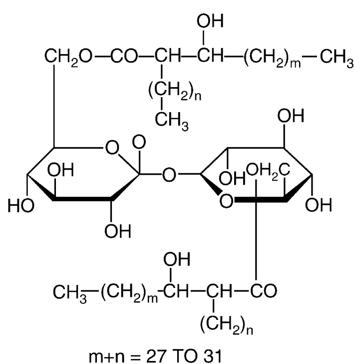
Biosurfactants are amphipilic compounds with both hydrophilic and hydrophobic moieties (mostly hydrocarbons) able to display a variety of surface activities that, among other roles, help the producing microorganisms to solubilise hydrophobic substrates (Desai and Banat, 1997). In recent years biosurfactants have been investigated as potential replacements for synthetic surfactants and may have many potential industrial and environmental applications (Kosaric, 1993; Banat et al., 2000). Biosurfactants represent a family of structurally diverse molecules with high and low molecular weights. This paper will concentrate on the low molecular weight biosurfactants, which for the purpose of this paper we aim to focus mainly on glycolipids consisting of carbohydrates joined to fatty acids or hydroxy-fatty acids chains. Low molecular lipopeptites analysis is mainly proteomic based and therefore will be discussed in the following chapter. There are four major groups of microbial glycolipids, rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids (➲ *Fig. 1*).

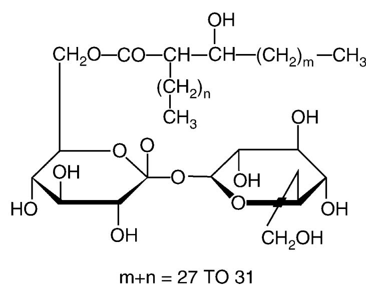
Rhamnolipids, produced by *Pseudomonas aeruginosa*, consist of either one or two rhamnose units connected to one or two β-hydroxy fatty acid chains with length of 8–12 carbons but with decanoic acid (C_{10}) being the most abundant (Desai and Banat, 1997). Sophorolipids consist of two major groups, the acidic sophorolipids comprising of a disaccharide, sophorose, linked to the sub-terminal or terminal carbon of the fatty acid chain and the lactonic sophorolipids where the carboxylic acid portion of the fatty acid is joined to carbon 4” of the disaccharide unit (Nunez et al., 2001). A large number of other variations in the structure of sophorolipids also occur (Asmer et al., 1988). Sophorolipids are most commonly produced by *Candida bombicola* and *Candida apicola*, along with a number of other species of this genus. Trehalose lipids are made up of a disaccharide, trehalose, linked by an ester bond to α-branched β-hydroxy fatty acids (Lang and Philp, 1998). The α-branched β-hydroxy fatty acids are connected at the C6 and C6′ of the carbohydrate structure in the case of the trehalose











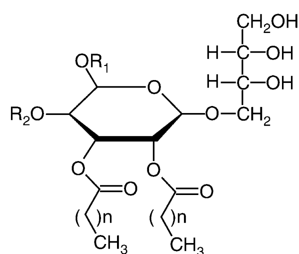


Figure 1

Chemical structures of microbial glycolipids.

dimycolates and at C6 for the monomycolates; other structure types have also been reported (Lang and Philp, 1998). The production of trehalose lipids is associated with most species of *Mycobacterium*, *Rhodococcus* and *Corynebacterium* (Asselineau and Asselineau, 1978). Finally, mannosylerythritol lipids (MELs) have four major structural groups and generally comprise 4-O-β-D-mannopyranosyl-D-erythritol connected to two medium length chains of fatty acyl esters (Fukuoka et al., 2007). MELs are generally produced by *Pseudozyma* yeasts species, *P. rugulosa*, *P. aphidis* and *P. antarctica*.

Chemical and structural analysis of microbial glycolipids can be carried out using a broad range of techniques, from simple colorimetric assays to sophisticated mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques. The methods used greatly depends on how much information is required, a recommended experimental procedure should rely on the following key-steps: (1) extraction of glycolipids from the culture medium, (2) detection; (3) purification and separation of the crude product (4) structural analysis, through the use of HPLC, MS and NMR.

A thorough identification as well as quantification of a glycolipid molecule would need a combined use of some or all of these resources together to overcome the further difficulties caused by the presence of various structural types or isomers of the same molecule.

The methods provided here represent the most common techniques that can be used for analysis of glycolipids. In some cases the experimental technique is the same for all four types of glycolipid, while for others it may vary greatly. Some of the techniques can be carried out without the need for complex equipment, while others, such as MS and NMR, use expensive equipment and require expertise to operate. This chapter is therefore intended to provide insights and suggestions on how to approach the analysis of microbial glycolipids. Further information on each procedure for analysis and information regarding interpretation of results can be found in the references listed.

2 Experimental Approach

2.1 Extraction of Glycolipids

The aim is to obtain a crude extract free from the aqueous culture medium. Although a variety of methods are available the most commonly used is solvent extraction.

2.1.1 Rhamnolipids

2.1.1.1 Acid Precipitation

1. Remove cells from culture broth by centrifuging at 13,000 × *g* for 15 min.
2. Acidify supernatant by dropwise addition of concentrated HCl, to pH 3.0 and maintain at 4°C for several hours.[1]
3. Centrifuge at 20,000 × *g* at 4°C for 20 min to obtain precipitate (Deziel et al., 1999).

[1] Under acidic conditions (pH 3.0), the rhamnolipids are present in their protonated form (pKa 5.6; Ishigami et al., 1987) and are therefore less soluble in water (Schenk et al., 1995).

2.1.1.2 Solvent Extraction

This extraction technique is used in combination with acid precipitation for more efficient yields.

1. Remove cells by centrifuge at 13,000 × *g* for 15 min.[2] Acidify by addition of concentrated HCl to pH 3.0 and transfer to a separating funnel.
2. Extract three times with an equal volume of ethyl acetate,[3] shaking vigorously each time and allow the two layers to separate in a separating funnel.
3. Transfer bottom aqueous layer and the top ethyl acetate layer to separate flasks. Re-extract the aqueous portion twice more or until no further color persists in the ethyl acetate layer.
4. Add 0.5 g of magnesium sulfate per 100 ml of ethyl acetate portion, to remove the traces of water present, filter and rotary evaporate to yield a brown gum extract.

2.1.2 Sophorolipids

2.1.2.1 Solvent Extraction

Extraction is carried out in similar manner to rhamnolipids, but without removal of cells or acidification. When high yields are produced in the fermentation, direct centrifuging to obtain sophorolipids free from the supernatant is also possible (Nunez et al., 2001).

1. Procedure as per Sect. 2.1.1.2. Steps 2–4
2. When ethyl acetate is removed, excess lipidic carbon source that is also extracted should be removed by washing three times with hexane.

When high yields are produced (>4 g l^{-1}), the procedure becomes much easier as sophorolipids are denser than water and would settle down after the centrifugation step (Nunez et al., 2001). The honey-like sophorolipid material at the bottom can be removed and washed several times with water.

2.1.3 Trehalose Lipids

2.1.3.1 Solvent Extraction

Extraction is carried out in similar manner to rhamnolipids, using the whole culture broth and without acidification (Rapp et al., 1979).

1. Extract three times with equal volumes of chloroform:methanol (2:1, v/v), shaking vigorously each time and allow the two layers to separate.[4]
2. Transfer bottom layer (organic solvent) to a flask and re-extract the aqueous portion twice more or until no further color is extracted. Remove the solvent by rotary evaporation.

[2] When larger volumes need to be extracted, autoclaving to sterilise without removal of cells may be an advantage.

[3] Chloroform:methanol (2:1, v/v) can also be used for extraction, however the time taken for the two layers to separate is much greater.

[4] MTBE has also been shown to be a suitable solvent for extraction of trehalose lipids (Kuyukina et al., 2001).

2.1.4 Mannosylerythritol Lipids (MELs)

2.1.4.1 Solvent Extraction

Extraction of MELs is carried out in the same way as sophorolipids.

1. Procedure as per Sect. 2.1.1.2. Steps 2–4.
2. When ethyl acetate is removed, excess lipidic carbon source that is co-extracted should be removed by washing three times with hexane.

2.2 Colorimetric Detection

Colorimetric methods can be used to determine the presence of the biosurfactants in either the culture medium or the extract. Detection can be carried out using assays detecting the sugar moieties such as anthrone (Hodge and Hofreiter, 1962) or orcinol assay without the need for extraction. However, interferences from chemicals and carbon sources can result in inaccurate results and therefore should only be used as a rough indicator of biosurfactant production.

2.2.1 Anthrone Assay

The anthrone assay can be used to detect and roughly quantify the amount of glycolipid present in the culture broth (Hodge and Hofreiter, 1962). This assay can be used for all types of glycolipid biosurfactants and detects the amount of carbohydrate present.

1. Prepare the anthrone reagent as follows; pipette 5 ml of absolute ethanol into 100 ml flask, add 200 mg of anthrone (9,10-dihydro-9-oxoanthracene) and make up to 100 ml with 75% sulfuric acid.
2. To assay add 200 µl of cell-free supernatant and 1,000 µl of anthrone reagent.
3. Heat in boiling water for 9 min.
4. Cool and measure absorbance at 625 nm.
5. Prepare a standard curve using a pure sample of the glycolipid being investigated at concentrations 0–50 µg ml^{-1} using the same procedure.

2.2.2 Orcinol Method

This colorimetric assay is based on the reaction of orcinol (1,3-dihydroxy-5-methylbenzene) and the sugar moiety under acidic conditions and high temperature to produce a blue-green colored dye whose absorbance can be measured and used to roughly quantify the glycolipid concentration in the sample (Koch et al., 1991).

1. Prepare the orcinol reagent containing 0.19% orcinol in 53% H_2SO_4. To prepare 20 ml, dissolve 0.038 g of orcinol in 9.4 ml distilled water, using heat if necessary. Cool in ice and add slowly 10.6 ml of concentrated H_2SO_4.

2. To 100 μl of cell-free supernatant add 900 μl of orcinol reagent at incubate at 80°C for 30 min. Allow mixture to cool to room temperature.
3. Measure the absorbance at 421 nm and prepare a standard curve using pure glycolipid at concentration 0–50 μg ml^{-1} using the same procedure.[5]

2.2.3 Thin Layer Chromatography Detection/Purification

Thin layer chromatography (TLC) is a simple method allowing detection of glycolipids and can also provide information on possible structural types of glycolipids present. TLC detection should be carried out before purification procedures to determine the presence of glycolipids and can also be used to determine purity after purification steps.

2.2.3.1 TLC of Rhamnolipids

TLC has been used for detection and composition of rhamnolipids in culture broth extracts (De Koster et al., 1994). Under normal phase TLC conditions monorhamnolipids and dirhamnolipids are separated into two bands. Preparative TLC can also be used to purify small quantities of rhamnolipids for analysis.

1. Dissolve a small quantity of crude extract in chloroform and apply 10 μl onto a TLC plate (silica gel 60) and apply at point of origin near the bottom of the plate.
2. Previously purified rhamnolipids should be applied as standards for comparison.
3. Once dried, develop plate in solvent system of chloroform:methanol:acetic acid (6.5:1.5:0.2, v/v/v) (Deziel et al., 2000).
4. When developed remove plate and allow to air-dry in a fume cupboard
5. Prepare anthrone reagent by mixing 63 ml of sulfuric acid, 25 ml of water and 0.125 g of anthrone, under ice conditions.
6. Spray the plate evenly with anthrone reagent and place in an oven at 110°C for 20 min.
7. On visualisation the spot (green color) nearer the point of origin corresponds to the dirhamnolipids, while the spot further from the point of origin represents the monorhamnolipids. On a preparative scale the bands can be scraped and extracted to yield purified rhamnolipids.

2.2.3.2 TLC of Sophorolipids

TLC for sophorolipids can be carried out as per Sect. 2.2.1.1 with the following modifications:

1. At step 2 use sophorolipid as standard
2. At step 3 use solvent of chloroform:methanol:water (6.5:1.5:0.2, v/v/v) (Asmer et al., 1988).
3. For detection use *p*-Anisaldehyde as the spray reagent in step 5. Prepared by mixing 100 ml acetic acid, 2 ml of sulfuric acid and 1 ml of *p*-anisaldehyde.
4. Compare spots obtained with published data (Asmer et al., 1988). Acidic sophorolipids appear near the point of origin while the lactonic forms appear above.

[5] Use a glycolipid standard appropriate to the biosurfactant under investigation.

2.2.3.3 Trehalose Lipids

TLC is carried out according to the procedure for sophorolipids at Sect. 2.2.3.2. Results can be compared with published data with trehalose monomycolates appearing near the point of origin with trehalose dimycolates slightly above (Kretschmer et al., 1982). Trehalose lipids will appear green using *p*-anisaldehyde. Other spots are likely to be detected corresponding to other components of the trehalose lipid extract.

2.2.3.4 TLC of MELs

Detection of MELs can be carried out in a similar manner to that used for rhamnolipids with the following modifications (Kitamoto et al., 1990a).

1. Use a previously purified sample of MELs as a standard and at step 3, develop plate in solvent system of chloroform:methanol: 7N ammonium hydroxide (6.5:1.5:0.2, v/v/v).
2. At step 7 compare with published data (Konishi et al., 2007) with separation from point of origin to solvent front in the order MEL D to MEL A.

2.3 Purification

Generally, a number of impurities are often co-extracted during extraction along with several structural types of the target biosurfactant, which are produced in varying quantities. These may need to be evaluated by separating and removing the impurities.

2.3.1 Silica Gel Column Chromatography

Column chromatography is a relatively inexpensive method that can be used to purify glycolipids. Using this technique milli-gram to kilo-gram quantities of glycolipids can be obtained free from impurities and can also be used to separate structural types of glycolipids for further analysis.

2.3.1.1 Rhamnolipids

Using this technique excess carbon sources such as fatty acids and other impurities that are co-extracted with the glycolipids can be removed (Itoh et al., 1971). Separation of both mono-rhamnolipids and dirhamnolipids can be carried out.

1. Use a clean glass chromatography column with porous support at the bottom.
2. Prepare silica gel 60 (200–425 mesh, 0.035–0.075 mm) in chloroform and stir to remove trapped air. The amount of silica required depends on the size of the column and quantity of material to be separated. Generally the amount of silica should be 20 times the sample weight, e.g., for 10 g sample use 200 g silica gel.
3. Pour slurry into column and pack tightly by continuous flow of chloroform. Once packed place 0.5 cm layer of sand (white quartz, mesh-50 + 70) on top.
4. Dissolve sample in a minimal volume of chloroform (20 ml) and add silica (25 g) until majority of solvent is absorbed, dry using rotary evaporation. Pour into column and add another layer of sand.

5. Wash column with chloroform (≈1.5 l) until no color persists, to remove neutral lipids and non-polar pigments.
6. Change solvent conditions to chloroform:methanol (5:0.3, v/v) followed by (5:0.5, v/v) and continue elution (1 l each) until the observed band corresponding to monorhamnolipids is eluted, collecting samples at 100 ml intervals.
7. Elute the dirhamnolipids with chloroform:methanol (5:5, v/v) (≈1 l).
8. Dry down each fraction and TLC to confirm presence of rhamnolipids. Combine fractions based on TLC profile to obtain pure monorhamnolipids and dirhamnolipids.

2.3.1.2 Sophorolipid

Purity of sophorolipids is usually reasonably high after the extraction process and column chromatography is generally used for separation purposes. A major difficulty in separating sophorolipids is that they range from the highly polar acidic forms to the relatively non-polar lactonic types. Therefore, the separation in acidic and lactonic types can be quite easily carried out, while purification of the ten different structural groups presents some more difficulties (Davilla et al., 1993). Several methods are available using column chromatography (detailed here) but each requires profiling of fractions by TLC to determine separation of structural groups. Other approaches utilizing medium pressure column chromatography (MPLC) (Asmer et al., 1988) and crystallization to separate lactonic from acidic forms (Hu and Ju, 2001a) have been also reported.

The method should be carried out according to Sect. 2.3.1.1 with the following modifications.

1. At steps 6 and 7 the elution conditions should be mixtures of chloroform and methanol. No set method is available for separation but generally starting at 9.8:0.2 v/v (chloroform: methanol) ranging to 6:4 will elute all sophorolipids present.[6]
2. For step 8, depending on the quantity of extract and size of column, equal volume fractions should be taken and analyzed by TLC and combined based on their profiles. Comparison with TLC method (Asmer et al., 1988) can help to tentatively identify the structural groups of sophorolipids.

2.3.1.3 Trehalose Lipids

The purification of trehalolipids 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 types of trehalolipids and a large number of other lipids along with excess *n*-alkane used as substrate in the production process complicates the purification process further. Consequently a preliminary column chromatography step to remove hydrocarbon residues is recommended before a subsequent column chromatography for the purification of trehalose lipids is carried out (Kretschmer et al., 1982).

Initial Column Chromatography 1

1. For removal of excess hydrocarbon substrates, set up column according to Sect. 2.3.1.1. Steps 1–5 with following modifications.

[6] Depending on how much separation is required, increments can range from 2% increases to 10% increases in methanol concentration.

2. At step two, use silica gel 60 with mesh size 70–230 (0.063–0.20 mm).
3. Use hexane instead of chloroform throughout steps 2–5.
4. By using hexane at step 5, the excess *n*-alkanes are moved
5. Elute trehalose lipid and other lipids with chloroform:methanol (2:1, v/v).
6. Remove solvent by rotary evaporation and proceed to the second column as described below.

Column Chromatography 2

The volume of each elution condition depends on the quantity being separated. Fractions obtained from each step should be monitored by TLC according to Kretschmer et al. (1982) to detect each component.

1. Carry out purification of trehalose lipid according to Sect. 2.3.1.1. Steps 1–4.
2. Perform elution conditions as follows; starting with chloroform for triglycerides
3. Change to chloroform:methanol (10:1 v/v) to elute 3-keto-2-allyl fatty acids and fatty alcohols.
4. Using chloroform:methanol:acetic acid (5:1:0. 01, v/v/v) for fatty acid and 3-hydroxy-2-allyl fatty acids.
5. Elute trehalose lipids using chloroform:methanol (5:1.5 v/v) and (5:2 v/v).

2.3.1.4 Mannosylerythritol Lipids

MELs can be separated using silica gel column chromatography (Kitamoto et al., 1990b).

1. Perform separation according to procedure in Sect. 2.3.1.1. Steps 1–8 modified in the following way.
2. At step four dissolve 3.2 g of MEL extract in 5 ml chloroform and apply to column
3. At step 5, wash column with 400 ml of chloroform:ethyl acetate (4:1, v/v) until no color persists, to remove neutral lipids and non-polar pigments.
4. For step 6, elute MEL A with 800 ml of chloroform:acetone (7:3, v/v), followed by MEL B with 600 ml of chloroform:acetone (6:4, v/v).
5. At step 7, change solvent conditions to chloroform:acetone (5:5, v/v) and elute MEL C and MEL D with 300 ml.
6. Dry down each fraction and TLC to confirm presence of MEL.

2.4 Analysis

Once glycolipids are isolated in pure form, analysis is required to both quantify and/or investigate structural features. Numerous techniques have been currently developed to analyze glycolipid biosurfactants working either on the intact molecule or by breaking down the structure into its fatty acid and carbohydrate components. Among these techniques, mass spectrometry offers the greatest amount of information with regard to purity and structural conformation.

2.4.1 Lipid Analysis

Most glycolipid biosurfactants vary greatly in the lipid portion, therefore its analysis provides detailed structural information that allows the identification of glycolipid structures.

The process involves hydrolytic cleavage of the link between the carbohydrate and lipid portions by hydrolysis and subsequent derivatization of the resulting fatty acid chains to fatty acid methyl esters analyzed by GC or GC-MS (Brandl et al., 1988).

2.4.1.1 Rhamnolipids

1. Take 4 mg of purified glycolipid and dry completely.
2. To this add 1 ml chloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid and heat at 100°C for 140 min.
3. Add 1 ml of distilled water and shake vigorously for 1 min. Leave to stand for phase separation to occur.
4. Remove bottom layer (chloroform layer) containing the fatty acid methyl esters and analyze by GC or GC-MS.
5. GC analyses can be carried out using a variety of columns such as DB-23 capillary column or supelco omegawax (30 M × 0.25 mm × 0.25 μm id).
6. Analytical conditions that can be used are; injector temperature 250°C, start oven program at 50°C, hold for 1 min then ramp at 40°C per min to 215°C and hold for 25 min (Monteiro et al., 2007).
7. Electron impact at 70 eV with scan range 50–450 Da and an injection volume of 1 μl.
8. Fatty acid methyl esters retention times increase with chain length and degree of separation.[7]

2.4.1.2 Sophorolipids

Several different methods have been reported, however methanolysis is the simplest and can be additionally coupled with derivatization for improved the signal (Davilla et al., 1993). Briefly, methanolysis reaction is used to isolate the hydroxy fatty acid portion of sophorolipids (Cavalero and Cooper, 2003) and convert them to hydroxyl acid methyl esters, which is the method reported here.

1. Take approximately 30–50 mg of sophorolipid extract and add 2 ml methanol containing 1% sulfuric acid and 1 ml of toluene containing a standard (2 mg ml^{-1} of either arachidic acid or dodecanoic acid).
2. Heat at 100°C for 40 min.
3. Extract reaction product in separating funnel twice with 5 ml of cyclohexane in 5 ml of presence of 50 g l^{-1} NaCl.
4. Dry cyclohexane layer (top layer) under a stream of nitrogen.
5. GC-MS conditions as follows, column RTX-5 (Restek) or carbowax type (Supelco).[8] Conditions here apply to RTX-5 MS column with a diameter of 0.25 mm. Set injection temperature to 275°C and column conditions starting at 65°C ramping at 10°C min^{-1} to 320°C and hold for 5 min.
6. Set MS scan range at 10–350 with the ion source temperature at 200°C.

2.4.1.3 Trehalose Lipids

GC-MS analysis should be carried out according to procedure in Sect. 2.4.1.1.

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[7] As well as a variety of columns that can be used for analyses, the other conditions such as run temperatures and times can also be varied.

[8] Other columns and consequently different GC-MS conditions can be used.

2.4.1.4 Mannosylerhthritol Lipids

GC-MS analysis should be carried out according to procedure in Sect. 2.4.1.1.

2.4.2 HPLC Analysis

HPLC is a method that allows the separation of glycolipids and when coupled with evaporative light scattering detector (ELSD) or mass spectrometry provides valuable information needed for the identification and quantification of glycolipids. HPLC-UV can also be used for analysis when the test compounds have been derivatised to *p*-bromophenacyl esters (Schenk et al., 1995; Mata-Sandoval et al., 1999). HPLC-UV and HPLC-ELSD both require comparison with retention times of standards to allow identification of the structure, however, the presence of isomers cannot be detected.

2.4.2.1 HPLC-UV of Rhamnolipids

1. Take a small sample of glycolipid and air dry
2. Dissolve in 1 ml water and add 1 ml acetonitrile containing 2-bromoaceto-phenone and triethylamine. Molar ratio should be 1:4:2 (glycolipid:2-bromoacetophenone:Et_3N).
3. Heat for 1 h at 80 C and filter through 0.22 μm syringe filter to remove particulate material.
4. Using gradient HPLC with UV detection set at 244 nm, connect HPLC column (C_{18} column 250 mm × 4.6 mm × 5 μm i.d.).
5. Prepare mobile phase A (CH_3CN) and B (3.3 mM H_3PO_4)
6. Gradient conditions should be set as follows; 50% A and 50% B for 3 min, then to 100% mobile phase A over 19 min and held for 5 min, followed a change to 50% A over 3 min and held for 10 min.[9]
7. Set flow rate at 1.0 ml min^{-1} with an injection volume of 50 μl.
8. Standard curves of previously purified glycolipids should be obtained to quantify samples based on peak area.

2.4.2.2 HPLC-ELSD of Rhamnolipids

ELSD allows the detection of glycolipids without the need for derivatization. Its works by measuring the scattering of photons (light) by particles of compounds that have been evaporated from the mobile phase

1. Set up isocratic HPLC-ELSD with Chromosphere PAH 100 mm column (Chromopack, Netherlands) (Noordman et al., 2000).
2. Prepare isocratic mobile phase consisting of Acetonitrile:water (55:45 v/v) with 0.03% trifluoroacetic acid.
3. Set flow rate at 0.5 ml min^{-1} and injection volume 500 μl.[10]
4. Standard curve of pure glycolipid should be used for quantification.

[9] Other gradient conditions or slight modifications should be used to achieve appropriate separation depending on the column selected.

[10] It is possible to use other columns and change the elution conditions.

2.4.2.3 HPLC-ELSD of Sophorolipids

Sophorolipid extracts contain a mixture of both acidic and lactonic types and can contain up to 40 different types including isomers. The resolution of all components is quite difficult particularly for the acidic structures, however with ELSD detection, good separation of the majority of compounds particularly the lactonic structures is achieved using the method detailed below (Davilla et al., 1993).

1. Set up isocratic HPLC-ELSD with Hypersil C18 or equivalent, 150 mm × 4.6 mm × 5 μm I.D. (Noordman et al., 2000).
2. Set gradient elution conditions with 2% acetonitrile and 98% water changing to 70% acetonitrile over 48 min.
3. Set flow rate at 1.0 ml min^{-1} and injection volume at 20 μl.[11]

2.4.2.4 HPLC-ELSD of Trehalose Lipids

At present no method is available for analysis of trehalose lipids by HPLC.

2.4.2.5 HPLC-ELSD of Mannosylerythritol Lipids

MELs are commonly analyzed using HPLC-ELSD with normal phase silica columns. Since the majority of MEL production is carried out using soybean oil as the carbon source, it is possible to apply the sample without removing impurities using normal phase separation. Separation of the main types of MELs into four individual peaks has been demonstrated (Rau et al., 2005).

1. Set up gradient HPLC-ELSD with a silica gel column (Nucleosil 100 A, 5 μm, 4.6 × 250 mm, Phenomenex, UK) (Rau et al., 2005).
2. Prepare gradient solvent system mobile consisting of 99% chloroform and 1% methanol changing to 100% methanol by 30 min.
3. Set flow rate at 1.0 ml min^{-1} and injection volume 500 μl.
4. Standard curve of pure glycolipid should be used for quantification.

2.4.3 Mass Spectrometry Analysis

Mass spectrometry (MS) represents a powerful method for analysis of glycolipids, providing detailed structural information on the molecular mass of the compounds under investigation. Tandem MS (MS/MS) results in the fragmentation of structures thus allowing the identification of individual isomers without the need for separation. Moreover, when combined with HPLC, it provides the most sensitive method for identification and quantification of glycolipids. A drawback, however is that it requires high level of purification as salts and free nonpolar lipids can induce suppression of ion signals under MS conditions. Glycolipids can be analyzed on all types of mass spectrometers, with electrospray ionization (ESI-MS) and matrix assisted laser desorption ionization (MALDI) described here. As with all MS equipment, the experimental technique will vary greatly depending on the manufacturer of the instrument

[11] It is possible to use other columns and change the elution conditions.

and software, therefore, only specific details on sample preparation and basic operation procedures are described below. Refer to software manuals for specific details on the operation of equipment.

2.4.3.1 ESI-MS Analysis of Glycolipids

Electrospray ionization provides excellent glycolipid ionization when used for direct infusion or HPLC-MS. Using this technique virtually no fragmentation occurs in the primary molecules under investigation. Ionized molecules are detected by a mass analyzer according to their mass to charge ratio (*m*/*z*) and can be fragmented using collision-induced dissociation (CID) to provide valuable information about each structure and their isomers. Using an LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) the following method was used in our laboratory for glycolipids analysis.

1. Set tune method according to the following conditions; syringe 5 μl ml^{-1}, nitrogen sheath gas and auxiliary gas at 20 and 35, respectively. Spray voltage to 4.5 kV, Capillary temperature at 250°C, capillary voltage to 47.0 V and tune lens offset to 40 V.
2. Use negative ion mode with scanning range 50–1,200 *m*/*z*.
3. Dissolve sample in methanol at a concentration between 0.01 and 0.5 mg ml^{-1}.
4. Load the sample solution into the syringe, with the fused silica sample tube attach the syringe. Place syringe in pump and press start.
5. Start scan.
6. Mass spectrum of the sample should appear after a few seconds, change to the appropriate file name and press start to acquire data.[12]
7. After 1 min, press stop button to finish acquiring data.
8. Stop scan.

2.4.3.2 HPLC-MS of Rhamnolipids

Coupling of HPLC with mass spectrometry provides an accurate method for glycolipid identification. HPLC is used to separate the glycolipids, while each is individually analyzed by MS or even tandem MS (MS/MS) as they are eluted from the column. The ability to perform CID experiments helps to identify and quantify isomers without the need for complete separation (Deziel et al., 1999, 2000).

1. Connect HPLC to LCQ quadrupole ion-trap mass spectrometry.
2. Adjust tune method nitrogen sheath gas to 65 to evaporate the higher volume of solvent entering the mass spectrometer. Other conditions as above.
3. Use HPLC with Luna C18 column (250 mm × 4.6 mm × 5 μm) (Phenomenex) connected.
4. Prepare mobile phase A (acetonitrile) and B (water) and start gradient elution with 30% A in 70% B and which was then raised to 70% mobile phase A after 50 min.
5. Set flow rate at 0.5 ml min^{-1} and injection volume of 20 μl min^{-1}.
6. Tandem MS carried out using data dependent scans with threshold of 1×10^5 ion and collision energy of 35.0 eV. MS/MS is performed on the most intense peak in each scan.

[12] Method shown refers to the LCQ with ESI-MS carrying Excalibur software; for different equipment or software consult the manufacturers' manuals.

2.4.3.3 HPLC-MS of Sophorolipids

Using HPLC-MS the complete profile of sophorolipids can be determined without the need for total separation, which is difficult to achieve. The identification can be carried out based on elution order and molecular weight of each sophorolipid, which can be compared with other studies reported in the literature (Davilla et al., 1993; Nunez et al., 2001; Hu and Ju 2001b; De Koster et al. 1995).

1. Analysis carried out according to Sect. 2.4.3.2 with following variations.
2. At step 3 use a Gemini (Phenomenex) C18 column (250 mm × 4.6 mm × 5 μm) which produces better separation than the Luna column.
3. Step 4 elution conditions should be 50% acetonitrile and 50% water changing to 70% acetonitrile over 50 min.
4. When analyzing results it is necessary to check for other closely related structures, which maybe obscured by large peaks. For example, where a major peak as been assigned a structure, search for the same structure with 1 less or one extra double bond in lipid chain, i.e., 2 Da higher or lower. This is due to the large peak masking minor components of the extract, especially for acidic sophorolipids.

2.4.4 MALDI Analysis for all Types of Glycolipids

MALDI is a soft ionization mass spectrometry technique that allows the identification of intact compounds. Basically, samples to be analyzed are mixed with a matrix and dried on a platform, onto which a laser is fired with various degrees of energy thus forming gaseous ions, which can then be observed in a time of flight analyzer (TOF).

1. Prepare the matrix, α-cyano-4-hydroxy cinnamic acid (αCHCA), which should be used for glycolipids. Prepare a 10 mg ml^{-1} solution of αCHCA in acetonitrile/Ultrapure water/trifluoroacetic acid (5:5:0.01 v/v/v) and vortex until dissolved.
2. Dissolve sample in methanol and mix 1 μl with 1 μl of matrix and apply to sample plate.
3. Insert plate into instrument, move plate to sample position and fire the laser.
4. If the signal is poor increase laser strength or move plate slightly.[13]
5. When a good spectrum is obtained, save as a data file with appropriate file name.

2.4.5 NMR Analysis

The previously reported methodologies allow the identification of molecular structure to quite a high extent though not completely. To achieve a full structural determination, NMR needs to be utilized and is the most powerful method able to identify functional groups as well as the position of linkages within the carbohydrate and lipid molecules. Using a series of NMR experiments the exact location of each functional group can be obtained and information about the structural isomers is also possible.

[13] Signal is severely influenced by the presence of salts and other impurities.

The glycolipids should be dissolved in dueterated chloroform and a series of 1D (^{1}H and ^{13}C) and 2D (such as COSY, ROSY, HMQC and HMBC) experiments should be carried out by NMR. Specific details with regard to the results for rhamnolipids (Monteiro et al., 2007), sophorolipids (Davilla et al., 1993; Asmer et al., 1988), trehalose lipids (Rapp et al., 1979; Kretschmer et al., 1982) and mannosylerythritol lipids (Kitamoto et al., 1990a) can be obtained from the literature.

3 Solutions and Materials

1. 3.1 Extraction – Separating funnel, rotatory evaporator; ethyl acetate (for rhamnolipids (RL), sophorolipids (SL), mannosylerythritol lipids (MEL)); chloroform and methanol (for trehalose lipids (TL); magnesium sulfate, filter funnel and filter paper (for RL, SL, MEL); concentrated HCl (RL); centrifuge (RL).
2. 3.2 Colorimetric detection – UV spectrometer, water bath, pipettes; specific for anthrone assay-anthrone, ethanol, sulfuric acid, water; specific for orcinol assay-orcinol, sulfuric acid, water, ice.
3. TLC – TLC chamber tank, TLC plates generally silica gel 60 or equivalent, spray reagent bottle, oven, methanol, chloroform; acetic acid (RL); water (SL, TL); 7 N ammonium hydroxide; p-ansialdehyde reagent (for SL, TL) anthrone reagent (for RL, MEL).
4. Silica gel column chromatography – Large glass column (size depends on quantity being purified), silica gel 60 (200–425 mesh), silica gel 60 (70–230 mesh for TL), sand (white quartz), rotatory evaporator, TLC plates, spray reagents as above; chloroform (for RL, SL, TL, MEL); methanol (for RL, SL, TL); hexane (for TL); acetone (for MEL).
5. Lipid analysis – GC-MS with appropriate column, water bath, separating funnel, rotatory evaporator; chloroform, methanol, sulfuric acid, distilled water (for RL, TL, MEL); methanol, 1% sulfuric acid, toluene, cyclohexane, sodium chloride (for SL).
6. HPLC-UV – All for rhamnolipid, HPLC utilizing UV detection with C18 column, water, acetonitrile, 2-bromoaceto-phenone, triethylamine, water bath, 0.22 μm syringe filters, 3.3 mM H_3PO_4.
7. HPLC-ELSD – HPLC with ELSD detection and appropriate column attached. HPLC grade acetonitrile, HPLC water (all for RL, SL, TL, MEL); trifluoroacetic acid (for RL).
8. ESI-MS – Quadrupole ion-trap mass spectrometer with electrospray ionization; HPLC methanol
9. HPLC-MS – Mass spectrometry as above with HPLC connected and appropriate columns, HPLC acetonitrile, HPLC water.
10. MALDI – Mass spectrometry with MALDI soft ionization, vortex, matrix (α-cyano-4-hydroxy cinnamic acid (αCHCA), acetonitrile and ultrapure water.
11. NMR – NMR equipment, dueterated chloroform and NMR tubes.

4 Time Considerations

Time required to perform some of the procedures illustrated (e.g., extraction and purification) can be affected by several factors such as sample volume or presence of residual production substrates (oils in particular).

1. Extraction – varies according to quantity of material to be extracted. Roughly 100 ml could be extracted and dried down in space of 2 h.
2. Colorimetric assay – 3 h.
3. TLC – 3 h.
4. Purification by silica gel column chromatography-depends on quantity to be purified but can range from 1 day to 2 weeks.
5. Lipid analysis – combined derivatization and GC-MS analysis 1 day.
6. HPLC-ELSD – around 1 h per sample.
7. ESI-MS – 10 min per sample, including cleaning steps.
8. HPLC-MS – between half hour to one and half per sample depending on the run time.
9. NMR – 1 h per experiment or at most 1 day per sample.

5 Trouble Shooting and Tips

1. Extraction – main problem is inefficient extraction. Take extra care when extracting and repeat extensively until no color is further extracted
2. Colorimetric assay – High yield can be erroneously obtained due to interference of other components of culture medium and carbohydrates in particular. Be aware when unexpectedly high product yields are obtained.
3. TLC – Spray reagents may show the presence of other spots (caused for instance by carbon source or fatty acids) additionally to glycolipids. Check the color of glycolipid spot compared to published data.
4. Purification – Difficulty in efficiently removing all impurities when oily carbon substrates are used in the culture broth. Process may need to be repeated.
5. Lipid analysis – Problems occur when either the fatty acid is not released from the glycolipid or sufficient derivatization may not have occurred. Further derivatization such as TMS derivatization may be required.
6. HPLC-ELSD – Problems can occur if large amounts of impurities are present thus affecting the column separation. Perform further purification steps before this analysis.
7. ESI-MS – Poor signals are observed in the presence of excess oily carbon sources as they can even severely suppress the signal in the mass spectrometer. Purify further before ESI-MS analysis.
8. HPLC-MS – Same as both ESI-MS and HPLC-ELSD.
9. NMR – Impurities will severely affect results, therefore glycolipid samples should be totally pure before analysis.

6 Research Needs

Microbial glycolipid biosurfactants have many advantages over chemically synthesized surfactants, such as lower toxicity and are more environmentally friendly, whilst providing similar surface activity. In our opinion, the major research needs surround the production of high yields and cost effective downstream processing. Downstream processing is probably the most expensive process for microbial glycolipids. The ability to obtain reasonably pure glycolipids from fermentation requires several extraction and purification steps. These steps are made

simpler by the use of highly pure carbon sources, such as oleic acid and alkanes. However, the use of these pure carbon sources is extremely expensive, as a result, oil sources such as sunflower oil and soybean would a great deal be more cost effective. Indeed, even more favorable would be the use of waste oil sources such as frying oils etc, which would also provide an environmental solution on how to recycle these waste products. These waste oils would be reasonably inexpensive to purchase and thus further drive down the cost of glycolipid production. However, the downside would be that these highly complex oil sources would be extremely problematic as far as purification is concerned. Generally the ability to remove all impurities of these carbon sources to leave highly purified glycolipids presents the greatest problems in this research field. Future work should be focused on the production of microbial glycolipids using inexpensive carbon substrates with the highest yields possible, combined with cost effective downstream processing methods. By focusing on these areas, microbial glycolipids would become more attractive as possible alternatives to commercial surfactants.

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