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Hydrocarbon and Lipid Microbiology Protocols

Biochemical Methods

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Preface to Hydrocarbon and Lipid Microbiology Protocols[1]

All active cellular systems require water as the principal medium and solvent for their metabolic and ecophysiological activities. Hydrophobic compounds and structures, which tend to exclude water, although providing *inter alia* excellent sources of energy and a means of biological compartmentalization, present problems of cellular handling, poor bioavailability and, in some cases, toxicity. Microbes both synthesize and exploit a vast range of hydrophobic organics, which includes biogenic lipids, oils and volatile compounds, geochemically transformed organics of biological origin (i.e. petroleum and other fossil hydrocarbons) and manufactured industrial organics. The underlying interactions between microbes and hydrophobic compounds have major consequences not only for the lifestyles of the microbes involved but also for biogeochemistry, climate change, environmental pollution, human health and a range of biotechnological applications. The significance of this "greasy microbiology" is reflected in both the scale and breadth of research on the various aspects of the topic. Despite this, there was, as far as we know, no treatise available that covers the subject. In an attempt to capture the essence of greasy microbiology, the *Handbook of Hydrocarbon and Lipid Microbiology* (http://www.springer.com/life+sciences/microbiology/book/978-3-540-77584-3) was published by Springer in 2010 (Timmis 2010). This five-volume handbook is, we believe, unique and of considerable service to the community and its research endeavours, as evidenced by the large number of chapter downloads. Volume 5 of the handbook, unlike volumes 1–4 which summarize current knowledge on hydrocarbon microbiology, consists of a collection of experimental protocols and appendices pertinent to research on the topic.

A second edition of the handbook is now in preparation and a decision was taken to split off the methods section and publish it separately as part of the Springer Protocols program (http://www.springerprotocols.com/). The multi-volume work *Hydrocarbon and Lipid Microbiology Protocols*, while rooted in Volume 5 of the Handbook, has evolved significantly, in terms of range of topics, conceptual structure and protocol format. Research methods, as well as instrumentation and strategic approaches to problems and analyses, are evolving at an unprecedented pace, which can be bewildering for newcomers to the field and to experienced researchers desiring to take new approaches to problems. In attempting to be comprehensive – a one-stop source of protocols for research in greasy microbiology – the protocol volumes inevitably contain both subject-specific and more generic protocols, including sampling in the field, chemical analyses, detection of specific functional groups of microorganisms and community composition, isolation and cultivation of such organisms, biochemical analyses and activity measurements, ultrastructure and imaging methods, genetic and genomic analyses, systems and synthetic biology tool usage, diverse applications, and

[1] Adapted in part from the Preface to *Handbook of Hydrocarbon and Lipid Microbiology*.

the exploitation of bioinformatic, statistical and modelling tools. Thus, while the work is aimed at researchers working on the microbiology of hydrocarbons, lipids and other hydrophobic organics, much of it will be equally applicable to research in environmental microbiology and, indeed, microbiology in general. This, we believe, is a significant strength of these volumes.

We are extremely grateful to the members of our Scientific Advisory Board, who have made invaluable suggestions of topics and authors, as well as contributing protocols themselves, and to generous *ad hoc* advisors like Wei Huang, Manfred Auer and Lars Blank. We also express our appreciation of Jutta Lindenborn of Springer who steered this work with professionalism, patience and good humour.

Colchester, Essex, UK — Terry J. McGenity
Braunschweig, Germany — Kenneth N. Timmis
Palma de Mallorca, Spain — Balbina Nogales

Reference

Timmis KN (ed) (2010) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Heidelberg

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About the Editors

Terry J. McGenity is a Reader at the University of Essex, UK. His Ph.D., investigating the microbial ecology of ancient salt deposits (University of Leicester), was followed by postdoctoral positions at the Japan Marine Science and Technology Centre (JAMSTEC, Yokosuka) and the Postgraduate Research Institute for Sedimentology (University of Reading). His overarching research interest is to understand how microbial communities function and interact to influence major biogeochemical processes. He worked as a postdoc with Ken Timmis at the University of Essex, where he was inspired to investigate microbial interactions with hydrocarbons at multiple scales, from communities to cells, and as both a source of food and stress. He has broad interests in microbial ecology and diversity, particularly with respect to carbon cycling (especially the second most abundantly produced hydrocarbon in the atmosphere, isoprene), and is driven to better understand how microbes cope with, or flourish in hypersaline, desiccated and poly-extreme environments.

Kenneth N. Timmis read microbiology and obtained his Ph.D. at Bristol University, where he became fascinated with the topics of environmental microbiology and microbial pathogenesis, and their interface pathogen ecology. He undertook postdoctoral training at the Ruhr-University Bochum with Uli Winkler, Yale with Don Marvin, and Stanford with Stan Cohen, at the latter two institutions as a Fellow of the Helen Hay Whitney Foundation, where he acquired the tools and strategies of genetic approaches to investigate mechanisms and causal relationships underlying microbial activities. He was subsequently appointed Head of an Independent Research Group at the Max Planck Institute for Molecular Genetics in Berlin, then Professor of Biochemistry in the University of Geneva Faculty of Medicine. Thereafter, he became Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI) and Professor of Microbiology at the Technical University Braunschweig. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, pioneered the genetic design and experimental evolution of novel catabolic activities, discovered the new group of marine hydrocarbonoclastic bacteria, and conducted early genome sequencing of bacteria that

became paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*). He has had the privilege and pleasure of working with and learning from some of the most talented young scientists in environmental microbiology, a considerable number of which are contributing authors to this series, and in particular Balbina and Terry. He is Fellow of the Royal Society, Member of the EMBO, Recipient of the Erwin Schrödinger Prize, and Fellow of the American Academy of Microbiology and the European Academy of Microbiology. He founded the journals *Environmental Microbiology, Environmental Microbiology Reports* and *Microbial Biotechnology*. Kenneth Timmis is currently Emeritus Professor in the Institute of Microbiology at the Technical University of Braunschweig.

Balbina Nogales is a Lecturer at the University of the Balearic Islands, Spain. Her Ph.D. at the Autonomous University of Barcelona (Spain) investigated antagonistic relationships in anoxygenic sulphur photosynthetic bacteria. This was followed by postdoctoral positions in the research groups of Ken Timmis at the German National Biotechnology Institute (GBF, Braunschweig, Germany) and the University of Essex, where she joined Terry McGenity as postdoctoral scientist. During that time, she worked in different research projects on community diversity analysis of polluted environments. After moving to her current position, her research is focused on understanding microbial communities in chronically hydrocarbon-polluted marine environments, and elucidating the role in the degradation of hydrocarbons of certain groups of marine bacteria not recognized as typical degraders.

Introduction

Hermann J. Heipieper

Abstract

Isolation of aerobic bacteria capable to degrade all different compounds such as aliphatic and mono- and polyaromatic hydrocarbons as well as their anthropogenic derivatives, e.g., chlorinated aliphatic and aromatic compounds, has been an important field of hydrocarbon microbiology. Here, biochemical methods were mainly applied in order to characterize enzymatic pathways responsible for the microbial degradation of these hundreds of different organic compounds. These techniques were also applied to characterize enzymes and pathways of anaerobic degradation of different natural and anthropogenic hydrocarbons. Meanwhile, the research focus as well as application of biochemical methods turned more toward monitoring and assessment of catabolic process, in situ, e.g., in hydrocarbon-contaminated soils and waters. In addition, present research focuses on synthesis of high-value products from cheap oil hydrocarbon derivatives by microbial catalysts.

This volume will summarize present biochemical techniques dealing with those different aspects of modern hydrocarbon microbiology.

Keywords: Aerobic biodegradation, Anaerobic biodegradation, Enzymes, Monitoring bioremediation, Pathways, Polyhydroxyalkanoates

In hydrocarbon microbiology, gaining isolates of aerobic bacteria capable to degrade all different compounds such as aliphatic and mono- and polyaromatic hydrocarbons as well as their anthropogenic derivatives, e.g., chlorinated aliphatic and aromatic compounds, was in focus during the last decades. Biochemical methods were mainly applied in order to characterize enzymatic pathways responsible for the microbial degradation of hundreds of different organic compounds. This research is close to be completed and will therefore only be represented in this volume by summarizing results on very important enzymes such as methane and alkane monooxygenase, which can also be important as biocatalysts for enzymatic transformations. On the other hand, within the last 30 years, techniques used for investigation of aerobic

An error in the chapter title has been corrected. Incorrect: "Introduction to Volume 6: Biochemical Methods", Correct: "Introduction".

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degradation were also applied to characterize enzymes and pathways of anaerobic degradation of different natural and anthropogenic hydrocarbons, respectively. Therefore, this volume will also address elucidation of anaerobic hydrocarbon biodegradation pathways and mass balances.

Meanwhile, research focus in the field, as well as biochemical methods applied, turned more toward monitoring and assessment of catabolic process in situ, e.g., in hydrocarbon-contaminated soils and waters. In addition, present research also focuses on the synthesis of high-value products from cheap oil hydrocarbon derivatives by microbial biocatalysis.

Another aspect of modern environmental microbiology is monitoring of bioremediation, mainly done by molecular biological methods. The molecular biological approach is supported by biochemical methods regarding identification of PAH-degrading pathways by phenotype-based methods that are of importance.

The major part of this volume will deal with one of the main aspects of future environmental microbiology, namely, the synthesis of high-value products from cheap hydrocarbon substrates or other recalcitrant products such as plastics by using specialized bacteria. We will provide protocols for the isolation of important biomolecules, such as lipopeptides, glycolipids, glycoproteins, and bioemulsifiers from different microbial sources.

In addition, the whole spectrum of sustainable, biodegradable substitutions of the enormous amount of around 300 million tons of plastics per year is of great interest for environmental biotechnology and therefore an issue discussed in this volume. One of the main carbon and energy storage compounds of most bacteria are polyhydroxyalkanoates (PHA). Also the aspect of downstream processing is of importance for an economically sound production of these bioplastics. Here, the easiest way would be to exclude the PHA articles out of the bacteria by nondisruptive methods. It is known for several years that also bacterial envelopes are as nonhomogeneously organized like eukaryotic ones. Also the outer membrane of Gram-negative bacteria as well as the cytoplasmic membrane of all bacteria consists of lipid rafts. These nonhomogeneously organization offers new aspects for very specific exclusion of biomolecules by certain molecular techniques. A protocol for the isolation of lipid rafts from bacterial membranes will be provided in the present volume.

Protocols for the Isolation and Analysis of Lipopeptides and Bioemulsifiers

Thomas J. Smyth, Michelle Rudden, Konstantina Tsaousi, Roger Marchant, and Ibrahim M. Banat

Abstract

High molecular weight biosurfactants are produced from a number of different bacteria and comprise lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structural types, many of which have yet to be fully characterised. Lipopeptide biosurfactants are cyclic peptides with varying attached lipid chains and are most commonly isolated from *Bacillus* and *Pseudomonas* strains. A great deal of research has been carried out on these cyclic lipopeptides, and their structures have been fully characterised. The ability to isolate, purify and characterise these structures is extremely important, providing detailed information with regard to different cultivation condition and biological activities. Similar methods can be used for both lipopeptides and biopolymers especially when attempting to determine their amino acid sequences. The experimental techniques used to isolate, purify and analyse these biosurfactants are widely varied from simple colorimetric assays giving an approximate indication of the type of compounds present to the more complex mass spectrometric techniques that provide information on molecular mass and structural features. Mass spectrometry provides essential information in the identification of these structures using sophisticated MS/MS analysis and software technologies. The use of Edman degradation can help confirm the results observed from de novo sequencing experiments, thus providing further confirmation of amino acid sequence of both lipopeptides and protein-containing biopolymers. This chapter details information on the most commonly reported techniques used to analyse these types of biosurfactants.

Keywords: Bioemulsifiers, Biosurfactants, Chemical analysis, Emulsan, Lipopeptides, Surfactin, Viscosin

1 Introduction

Biosurfactants are amphiphilic compounds with both hydrophilic and hydrophobic moieties (mostly lipids) able to display a variety of surface activities that, among other roles, help solubilise hydrophobic substrates [1]. During the last decade, they have been under investigation as potential replacements for synthetic surfactants and are expected to have many potential industrial and environmental applications [2–5]. They generally can be grouped either as low or high molecular weight biosurfactants, the former consisting of

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glycolipids and lipopeptides and the latter of high molecular weight polymeric biosurfactants [6, 7]. The main focus of this chapter is the analysis of lipopeptides and high molecular weight biosurfactants using chemical and analytical techniques including proteomic-based techniques.

Lipopeptides are cyclic structures generally produced by *Bacillus* and *Pseudomonas* species. They mainly consist of hydrophilic peptides, usually between seven and ten amino acids long, linked to a hydrophobic fatty acid structure. *Bacillus* cyclic lipopeptides consist of three major groups known as the surfactin, iturin and fengycin families. Surfactin (Fig. 1a) is the most commonly studied lipopeptide, and its structure consists of a 7-amino acid cyclic sequence connected to C13–C16 fatty acids [8]. Iturin also consists of seven amino acids, though different to surfactin, linked to C14–C17 fatty acids, while fengycin is composed of ten amino acids with fatty acid chain lengths of C14–C18 [9]. *Pseudomonas* lipopeptides consist of a fatty acid attached to a peptide, which is cyclised to form a lactone ring between two amino acids in the peptide chain. *Pseudomonas* cyclic lipopeptides fall into four distinct classification, namely, the widely studied viscosin (Fig. 1b) and amphisin groups along with tolaasin and syringomycin families [10]. The viscosin group's general structure consists of a nine-amino acid peptide linked to a 3-hydroxy fatty acid, usually decanoic acid, while amphisin has an 11-amino acid peptide also linked to 3-hydroxy fatty acid [11]. A number of other *Pseudomonas* lipopeptides, not belonging to any of these families, have also been characterised, such as putisolvin I and II [12].

High molecular weight biosurfactants are generally grouped together as polymeric biosurfactants. They are produced by a number of different bacteria and are composed of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structural types [13]. The most commonly studied biopolymers are Emulsan and Alasan isolated from *Acinetobacter* species. Emulsan (Fig. 1c) is a lipopolysaccharide isolated from *Acinetobacter calcoaceticus* RAG-1 ATCC 31012 and has a molecular weight of around 1,000 kDa [14], while Alasan is a complex of an anionic polysaccharide and a protein with a molecular weight of around 1,000 kDa isolated from *Acinetobacter radioresistens* [15]. A number of other polymeric biosurfactants have been discovered but remain partially or totally uncharacterised.

Chemical and structural analysis of lipopeptides and high molecular weight biosurfactants is carried out using a broad range of techniques varying from simple colorimetric assays to sophisticated mass spectrometry (MS) and sequencing techniques [16]. Due to their size and structural complexity, biopolymers are much more difficult to characterise than the lower molecular weight lipopeptide biosurfactants. After isolation and purification procedures, molecular mass determination of the compounds of interest may be

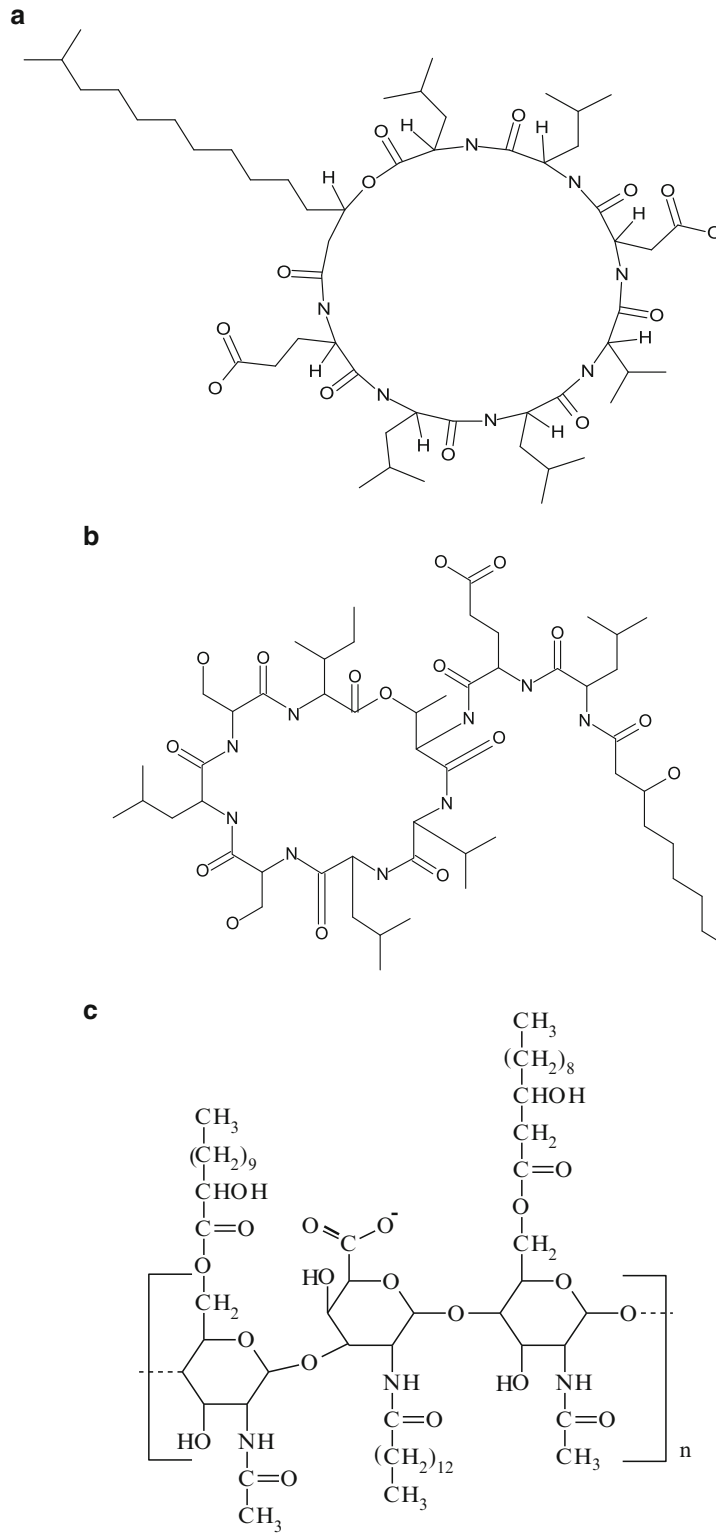

Fig. 1 The structure of cyclic lipopeptide surfactin (**a**) from *Bacillus* sp., viscosin (**b**) from *Pseudomonas fluorescens* SBW25 and a high molecular weight biopolymer Emulsan (**c**) from *Acinetobacter calcoaceticus* RAG-1

facilitated by mass spectrometry. This should be followed by analysis of the fatty acid portion using gas chromatography–mass spectrometry (GC-MS) and determination of the peptide sequence using the automated Edman degradation sequencing and a number of mass spectrometric techniques. This combined approach would provide the necessary information required for complete structural identification.

The analysis of biopolymeric biosurfactants presents a number of challenges due to their high molecular mass. Generally, the identification of the relative percentage of the carbohydrate, lipid and protein portions is carried out using simple colorimetric assays, such as the Bradford assay for protein determination and the phenol–sulphuric acid method for carbohydrates. This information is particularly useful when the biopolymer has not been previously characterised, which is normally the case, as it provides information on the techniques required for structural determination. For example, if the biopolymer contains only lipids and proteins, it is not necessary to carry out further analysis for polysaccharides. Determination of the approximate molecular mass can be carried out using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), gel permeation chromatography and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). However, for more detailed structural analysis to determine the exact composition requires the molecule to be broken down into more manageable portions. Removal of the carbohydrate and fatty acid portions means that these compounds can be analysed by gas chromatography–mass spectrometry (GC-MS) after derivatisation. Structural analysis of the protein portion of biopolymers requires its digestion into smaller peptides by enzymatic degradation. In this way, the individual sequences of the smaller cleaved peptides may be carried out using Edman degradation and mass spectrometric techniques. The information obtained can then be used to piece together the full protein structure.

The methods described here represent a variety of common techniques that can be used for analysis of lipopeptides and high molecular weight biosurfactants. Due to the less complex nature of lipopeptides, techniques such as SDS-PAGE and digestions are not required for their analysis. In contrast, these techniques are essential for polymeric biosurfactants allowing the smaller components obtained after digestion to be analysed in a similar manner to lipopeptides. 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 specific expertise. This chapter is therefore intended to provide insights and suggestions on how to approach the analysis of these biosurfactants. Further information on each procedure for analysis and information regarding interpretation of results can be followed up in the relevant literature cited.

2 Solutions and Materials

2.1 **Extraction of *Bacillus* lipopeptides**: concentrated HCl, methanol and filter paper.

2.2 **Organic solvent extraction of lipopeptides**: separating funnel, ethyl acetate, magnesium sulphate and filter paper.

2.3 **Lipoproteins and polymeric extraction: (for Ammonium sulphate precipitation)** ammonium sulphate, deionised water, hexane, dialysis tubing with molecular weight cut-off point of 5 kDa and dialysis clamps. For a**lcohol precipitation** or ethanol precipitation, ethanol.

2.4 **TLC of lipopeptides**: TLC plates (silica gel 60), chloroform, methanol, distilled water, fine-point capillaries and concentrated sulphuric acid for standard reagent spray, ninhydrin for amino acid detection and bromothymol blue for lipid detection.

2.5 **HPLC-UV/HPLC-MS for purification of different types of peptides/lipopeptides:** HPLC system connected to either UV or MS detector or both, a semi-preparative HPLC column such as reverse phase C18 or equivalent, HPLC water, acetonitrile and formic acid.

2.6 **Flash chromatography/column chromatography for large-scale purification of lipopeptides:** reverse phase C18 flash column, methanol, HPLC water and acetic acid.

2.7 **Fractionation of bioemulsifiers using fast protein liquid chromatography** (FPLC): FPLC column, 0.1 M PBS buffer.

2.8 **Gel permeation chromatography (GPC) fraction of bioemulsifiers:** GPC column, HPLC water and sodium nitrate.

2.9 **One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE):** double-distilled water, 1.5 M Tris–HCl at pH 6.6, acrylamide/Bis-acrylamide (30/0.8%), SDS, ammonium persulphate and TEMED (N',N',N',N'-tetramethylethylethylenediamine). For stacking gel, same as for running gel except 0.5 M Tris–HCl at pH 6.8. For the running buffer, glycine, Tris–HCl and SDS can be used. Sample buffer (Laemmli), bromophenol blue, 0.5 M Tris–HCl at pH 6.8, glycerol, SDS and double-distilled water. Other reagents include water saturated with butanol, filter paper, molecular weight marker, beta-mercaptoethanol, Coomassie Brilliant Blue stain, destain containing methanol water and acetic acid.

2.10 **Bradford assay for protein determination:** Coomassie Brilliant Blue, methanol, phosphoric acid, deionised water, BSA, sample tubes and spectrophotometer.

2.11 **Lowry method for protein determination:** BSA standard, Folin–Ciocalteu reagent, Modified Lowry Reagent and deionised water.

2.12 **Fatty acid analysis by GC-MS**: GC-MS equipment with GC column such as DB-23 capillary column or Supelco Omegawax. **Acid methanol method**: methanol, sulphuric acid, hexane and deionised water. **Boron trifluoride method**: sodium hydroxide, 25% boron trifluoride, methanol, deionised water and hexane.

2.13 **Colorimetric assay (phenol–sulphuric acid) for carbohydrate determination:** methanol, HCl, phenol and sulphuric acid.

2.14 **GC-MS determination of carbohydrate portion**: methanol, sulphuric acid, hexane, deionised water and TMSI (*N*-trimethylsilylimidazole)

2.15 **Protein digestion with proteases:** ammonium bicarbonate, ultrapure water, acetonitrile, Tris–HCl at pH 7.6, calcium chloride, DTT (dithiothreitol), iodoacetamide, formic acid and trypsin.

2.16 **MALDI-TOF mass spectrometry:** matrix α-cyano-4-hydroxy cinnamic acid (αCHCA) for lipopeptides/peptides, sinapinic acid for large molecular weight biosurfactants, acetonitrile and ultrapure water.

2.17 **Edman degradation:** Edman degradation sequencer and associated solvents and chemicals, acetonitrile and ultrapure water.

2.18 **Capillary chromatography with ion trap/QTOF MS/MS for de novo sequencing:** appropriate capillary liquid chromatography and connected to either an ion trap or QTOF mass spectrometry, ultrapure HPLC water, HPLC acetonitrile and formic acid.

2.19 **HPLC with QTOF MS/MS for de novo sequencing**: C18 HPLC column, HPLC water, acetonitrile and formic acid.

2.20 **NMR analysis of lipopeptides**: NMR equipment, deuterated chloroform and NMR tubes.

3 Experimental Approach

3.1 Extraction

The aim is to obtain a crude extract free from aqueous culture medium. Generally, the levels of lipopeptides and large molecular weight biosurfactants produced by the different species are in low quantities; therefore, the initial aim is to obtain a crude extract with enriched levels of the desired product.

3.1.1 Bacillus Lipopeptides

Extraction of lipopeptides is regularly carried out using a combination of acid precipitation and solvent extraction [17], with many slight variations in either the pH for precipitation, the organic

solvent used or the times reported for precipitation of the lipopeptides after acidification [18, 19].

1. To remove cells, centrifuge at 13,000×*g* for 15 min. at room temperature.
2. Acidify by the addition of concentrated HCl to pH 2.0, and allow precipitate to form at 4°C overnight.
3. Centrifuge at 13,000×*g* for 15 min at 4°C to obtain pellet.
4. Remove supernatant and extract the pellet three times with methanol.
5. Filter methanol to remove the remaining material and evaporate to dryness using rotary evaporation.

3.1.2 Organic Solvent Extraction of Lipopeptides

The extraction method reported above could be used for extraction of *Pseudomonas and Halobacteriaceae* lipopeptides; however, the most commonly reported method for this group of lipopeptides is solvent extraction with ethyl acetate [12, 20, 21].

1. Extract three times with an equal volume of ethyl acetate (*see* **Note 1**) shaking vigorously each time, and allow the two layers to separate in a separating funnel.
2. Transfer bottom aqueous layer and the top ethyl acetate layer to separate flasks. Re-extract the aqueous portion twice more or until no further colour persists in the ethyl acetate layer.
3. Add a small amount of magnesium sulphate to the ethyl acetate portion to remove the traces of water present, filter and rotary evaporate to yield a brown gum extract.

3.1.3 Lipoproteins and Polymeric Extraction

Extraction of the majority of high molecular weight biosurfactants is carried out using ammonium sulphate precipitation, followed by dialysis to remove any small molecules that may be present [14]. The methods reported for isolation of the high molecular biosurfactants are quite varied and generally specific to the actual biosurfactant present. Ammonium sulphate precipitation can be used for the majority of extractions particularly for biosurfactants with high protein contents. Nowadays, the more commonly applied method for high molecular weight biosurfactants, containing high levels of carbohydrate, is alcohol precipitation and with or without prior acidification may suffice. However, for more specific details, refer to related literature of the microorganism under investigation. Other techniques for high molecular weight biosurfactant isolation include TCA/acetone precipitation, acid/ethanol and chloroform/methanol. In each case, dialysis is usually performed on the crude product to remove low molecular weight impurities along with salts formed during the extraction procedure.

Ammonium Sulphate Precipitation

The ammonium sulphate precipitation method below is based on using 100 mL of culture broth and should be adjusted according to the starting volume.

1. Take 100 mL of culture broth and remove cells by centrifuging at 10,000×*g* for 15 min.
2. Cool the supernatant at 4°C, and add slowly while stirring 23.34 g of ammonium sulphate to obtain a 40% saturation solution (*see* **Note 2**). Leave at 4°C overnight.
3. Centrifuge at 10,000×*g* for 15 min to obtain pellet.
4. Resuspend in a 40% solution of ammonium sulphate and centrifuge again at 10,000×*g* for 15 min to obtain pellet.
5. Dissolve pellet in 20 mL water and extract with equal volume of hexane three times in a separating funnel to remove residual nonpolar lipids.
6. Using dialysis tubing with a molecular weight cut-off point of 5 kDa, clamp one end and rinse the tubing with distilled water to check for leaks.
7. Fill tubing with product from step 5 and clamp top. Place in beaker with dialysis buffer of distilled water, and place on a stirring plate in a cold room overnight.
8. Change dialysis buffer once after 6 h.
9. Remove sample after completion of dialysis and lyophilise to obtain biosurfactant product.

Alcohol Precipitation

Extraction of bioemulsifiers is now regularly carried out using ethanol precipitation with many slightly varying methods reported [22–24]. This method is best employed for bioemulsifiers with high carbohydrate content as it results in less binding of salts to the polysaccharide, which would occur with the ammonium sulphate precipitation method. The method below is based on the study by Franzetti et al. [22] and provides a general approach that can be applied to unknown bioemulsifiers.

1. Centrifuge at 7,000×*g* for 20 min at room temperature.
2. Freeze-dry the supernatant.
3. Reconstitute the dried product in deionised water and add ethanol (four times the volume of water) and allow precipitate to form at 4°C overnight.
4. Centrifuge at 7,000×*g* for 30 min at 4°C to obtain pellet (*see* **Note 3**).
5. Dry the remaining methanol from the pellet using nitrogen.
6. Dissolve in minimal amount of deionised water and dialyse using dialysis tubing for 24 h (any molecular weight cut-off between 6 and 12 kDa can be used).
7. After 24 h, remove the dialysate and repeat with fresh deionised water for a further 24 h.
8. After completion of the dialysis steps, freeze the retentate.

3.2 Detection/ Purification

After the initial extraction process, the crude lipopeptides or bioemulsifier usually contains a number of impurities, which are often co-extracted together with the target biosurfactant. Following extraction, the first major step is to determine the presence of the desired lipopeptides or bioemulsifier in the crude extract. The next step in analysis is determining the presence of impurities that require removal prior to further structural characterisation.

3.2.1 TLC of Lipopeptides

Thin-layer chromatography (TLC) is a common method used to detect the presence of lipopeptides [21, 25], while preparative TLC can be used to purify small quantities [26]. Spray reagents may show the presence of other impurity spots in addition to the lipopeptides. In this case, a second TLC should be carried out and ninhydrin spray reagent used to confirm the presence of amino acids.

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. Once dried, develop plate in solvent system of chloroform–-methanol–water (65:25:4) (*see* **Note 4**).
3. When developed, remove plate and allow to air-dry in a fume cupboard.
4. Spray the plate evenly with a solution of 5% sulphuric acid, and place in an oven at 110°C for 20 min to visualise spots.
5. If using ninhydrin, prepare a 0.25% solution in acetone, and spray plate evenly and heat at 110°C for 20 min to visualise spots.
6. Similarly detection of lipids can be carried out by spraying 0.1% bromothymol blue in 10% aqueous ethanol.
7. For preparative scale, remove lipopeptide spots by scraping the silica from plate into a flask and extract with chloroform–-methanol (2:1) overnight.
8. Filter methanol and rotary evaporate to dryness.

3.2.2 HPLC-UV/HPLC-MS for Purification of Different Types of Peptides/ Lipopeptides

HPLC is an excellent method for the separation of individual peptide/lipopeptide biosurfactants [27]. The most commonly employed technique is reversed phase chromatography, which results in the separation of each peptide/lipopeptide structure based on polarity. The separated products can be detected by UV absorbance detection and each individual peak collected using a fraction collector for further analysis of their structure. However, lipopeptides/peptides can co-elute due to their similar chemical properties which can be difficult to detect with UV, and coupling of HPLC with a mass spectrometer provides preliminary

information on the molecular mass of each component. Therefore, HPLC-MS is much more useful for determining whether co-elution is occurring, and once detected this problem can be overcome by modifying the elution conditions or changing the HPLC to a different column chemistry allowing the method to be transferred back to HPLC-UV.

For the purpose of this report, the basic steps in semi-preparative HPLC-MS are shown below, but equally semi-preparative HPLC with UV detection is commonly reported, and again a range of different methods can be found in the literature with analytical, semi-preparative and preparative HPLC methods for purification of lipopeptides [25, 28, 29]. In this case, mass spectrometry is carried out using a quadrupole ion trap (QIT) mass spectrometry, but the same separation procedure can be performed using other MS instruments such as a single quadrupole.

1. Interface HPLC to electrospray ionisation QIT mass spectrometry (ESI-MS) system.
2. Use semi-preparative C18 HPLC column (250 × 10 mm id), which can be purchased from a range of different suppliers (*see* **Note 5**).
3. Prepare the sample by dissolving in water. Centrifuge at 13,000 rpm for 5 min to remove particulate matter.
4. Prepare mobile phase A (water/formic acid (99.95:0.05, v/v)) and B (acetonitrile/formic acid (99.95:0.05, v/v)), and perform gradient elution starting with 70% A, 30% B changing to 0% A, 100% B over 40 min (*see* **Note 6**).
5. Set flow rate at 2.0 mL/min and injection volume of 500 μL/min.
6. Connect fraction collector, and set up the effluent flow from the column so that 90% is fraction collected and the remaining 10% sent to the MS detector.
7. Collect fractions at a rate of 2 mL/min. Fraction collection may be automated or peak collection may also be used.
8. MS scan range should be set at m/z 150–2,000.
9. MS/MS fragmentation can also be set up on the eluent entering the mass spectrometry. The information obtained can then be used to search a number of databases.
10. The individual fractions containing the lipopeptides can then be subjected to QTOF MS/MS de novo sequencing or Edman degradation to obtain sequence information on each individual peptide/lipopeptide.

3.2.3 Flash Chromatography/Column Chromatography for Large-Scale Purification of Lipopeptides

During the extraction procedures, a number of impurities are often co-extracted including partially constructed lipopeptides. The type of impurity and their quantity can have negative effects on the subsequent chemical characterisation. Furthermore, it is often necessary to study the bioactive or surface active properties of the various types of biosurfactant present in an extract by separating the different structural components. Column chromatography is a relatively inexpensive method regularly used to purify glycolipids. Using this technique, milligram to kilogram quantities can be obtained free from impurities and at the same time result in the generation of relatively pure lipopeptide products. These column chromatographic techniques can range from simple open glass columns to automated flash chromatography systems both of which have been used for the purification of lipopeptides [18, 29] with extremely different elution conditions. Similarly, using the same gradient conditions detailed below for flash chromatography [29], solid-phase extraction (SPE) cartridges are a cheaper alternative method. This method should provide some fractionation of the lipopeptides on a large scale; however, further prep or semi-preparative HPLC may be required for total purification of individual lipopeptide structure.

1. Condition a normal-phase silica column with a column bed of around 50 g (dependent on the sizes manufactured by each supplier) with 80% methanol (containing 0.1% acetic acid) for three column volumes. Generally the size of the column should be at least 20 times the sample weight, e.g. for 10 g sample use 200 g silica gel.
2. Prepare loading sample by dissolving 1 g of crude extract in methanol, and mix with 5 g of silica to produce slurry. Dry with a rotary evaporator to obtain a dried silica powder containing the crude extract.
3. Load dry material into the sample loading tube (specific to each flash chromatography manufacturer).
4. At a flow rate of 18 mL/min, ran 80% methanol (containing 0.1% acetic acid) through the column for 10 min.
5. Then elute by changing the gradient from 80 to 100% over 20 min and then for a further 20 min at 100% methanol (containing 0.1% acetic acid).
6. Collect each minute separately and monitor using UV at 210 nm.
7. Purification of fractions can then be determined by HPLC-MS.

3.2.4 Fractionation of Bioemulsifiers Using Fast Protein Liquid Chromatography (FPLC)

FPLC is a form of liquid chromatography specifically used for the purification of proteins and has been applied sporadically for the purification of bioemulsifiers [30, 31]. Since the procedure is based on the charge of protein in relation to the running buffer, the

presence of other components particularly carbohydrates containing anionic charges can interfere with this process. However, it still could be a useful tool for the purification of bioemulsifiers containing high protein content. The method below is a very generic method that could be adapted for different types of bioemulsifiers if required.

1. Condition a Sephacryl S-200 column (1.6 × 57 cm, Pharmacia K16/70 column [30]) with deionised water, and apply crude bioemulsifier dissolved in water.
2. Start the elution using 0.1 M PBS buffer.
3. Using a flow rate of 1.0 mL min, collect fractions every 2 min.
4. Monitor the elution at 280 mn to confirm the presence of protein-containing bioemulsifier.

3.2.5 Gel Permeation Chromatography (GPC) Fraction of Bioemulsifiers

GPC is a type of size exclusion chromatography that can be used to fractionate bioemulsifiers. Unfortunately, it is rarely reported for this compound group probably due to the difficulties that would be encountered when analysing a polymer that is neither fully protein nor fully carbohydrate. GPC is regularly used to determine the average molecular weight of proteins and polysaccharides; it would be difficult to achieve a reliable molecular weight for bioemulsifier. This is mainly due to the fact that size exclusion chromatography can also display other chromatographic mechanisms such as ion exchange effects. For this reason, pH and ionic strength of the mobile phase buffer is critical. Since bioemulsifiers are mostly composed of a significant proportion of protein and carbohydrate incorporated into its structure, the choice of buffer to ensure that only size exchange mechanisms are observed becomes extremely difficult. Furthermore, when analysing protein and polysaccharides, protein standards and pullulan polysaccharides standards can be used to generate and external molecular weight curve, which is then used to obtain unknown. To date, no such standards are available for bioemulsifiers or indeed any other mixed polymer. However, GPC still has advantages in that it can be used to purify and fractionate complex bioemulsifier due to the fact that separation based on molecular size will still occur albeit without the ability to accurately determine the molecular weight.

1. Set up GPC column (Waters Ultrahydrogel column or equivalent for aqueous-based size exclusion) on a standard HPLC system with a refractive index (RI) detector [23] (*see* **Note 7**) and fraction collection capabilities.
2. Prepare mobile phase of HPLC water containing 0.25 mM/L sodium nitrate, sonicate and filter prior to use.
3. Set a flow rate of 0.6 mL/min and condition for several hours.
4. Inject 50 μL per sample and set up fraction collector to collect 0.6 mL/min when peaks are detected.

3.2.6 One-Dimensional Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)

SDS-PAGE is commonly used for proteomic experiments and is extremely useful for the separation of protein mixtures (*see* **Note 8**) and for estimating the molecular mass of high molecular weight biosurfactants/proteins. The proteins are applied to one end of a gel in a loading buffer that contains a reducing buffer and SDS. The reducing buffer is used to cleave the disulphide bonds rendering the proteins in a linear form. SDS attaches to the protein relative to its molecular mass, resulting in a net negative charge on the protein. When a voltage is applied to the gel, proteins separate according to molecular mass. The individual bands can then be removed and the proteins extracted [31].

1. Prepare 10% running gel (*see* **Note 9**) using the following: 12.3 mL of double-distilled water, 7.5 mL of gel buffer which is 1.5 M Tris–HCl at pH 8.8 (6 g of Tris in 100 mL of water adjusted with NaOH), 9.9 mL of acrylamide/Bis-acrylamide (30/0.8% w/v), 0.3 mL of 10% SDS (w/v), 0.15 mL of 10% ammonium persulphate (APS w/v) and finally 0.01 mL of TEMED (*N*′,*N*′,*N*′,*N*′-tetramethylethylethylenediamine). For the last two parts (APS and TEMED) of the running gel, add just before pouring the gel.
2. For the preparation of stacking gel, use the following: 3.05 mL of double-distilled water, 1.25 mL of stacking buffer (0.5 M Tris–HCl at pH 6.8), 0.665 mL of acrylamide/Bis-acrylamide (30/0.8% w/v), 50 μL of 10% SDS, 50 μL of 10% APS and 5 μL of TEMED (*see* **Note 10**). For the last two parts (ABS and TEMED) of the stacking gel, add just before pouring the gel.
3. Running buffer should be prepared as follows: 200 mM glycine, 25 mM Tris–HCl at pH 6.8 and 0.1% (w/v) SDS.
4. Prepare Laemmli sample buffer [32] by using 0.02 g (w/v) of bromophenol blue, 0.313 mL of stacking buffer (0.5 M Tris–HCl at pH 6.8), 0.5 mL 10% (w/v) SDS and 0.25 mL (v/v) glycerol, and make up entire solution to 2.5 mL with double-distilled water.
5. Set up gel electrophoresis as per standard procedure, and pour the running gel prepared in step 1 with APS and TEMED being added just before pouring. Place 1 mL of water saturated with 2-propanol carefully on top of monomer solution, and leave for 45 min to allow the gel to polymerise.
6. Pour the overlying water off and drain the excess with strips of filter paper.
7. Add the stacking gel buffer with APS and TEMED being added just before pouring. Insert sample comb at an angle to allow air bubble to escape, and leave to set for 15 min.
8. Remove the comb and place gel in the buffer chamber and add running buffer to cover the gel.

9. Prepare the protein sample to be investigated by adding 20 μL of proteins to 20 μL of sample buffer (step 4) containing 1 μL of beta-mercaptoethanol (*see* **Note 11**) (used to reduce the disulphide bonds). Incubate at 95°C for 10 min.
10. Load 5 μL of the molecular weight marker, prepared with sample buffer from step 9, and 10 μL of each sample into separate wells.
11. Put on lid and connect power supply with 200 volts and run the gel. Stop the electrophoresis when the marker dye is less than 1 cm from bottom of plate (~1 h).
12. Remove gel and immerse in Coomassie Brilliant Blue stain (*see* **Note 12**) (made by adding 1.25 g of Coomassie R-250, 250 mL methanol, 50 mL of acetic acid and 200 mL of water) and leave overnight.
13. Pour off the Coomassie stain and destain using 50% methanol in water containing 10% acetic acid. Cover gel with destain solution and leave for 20 min, and then remove and repeat for a further 10 min.
14. Record image of gel, and determine approximate molecular mass of each protein or high molecular weight biosurfactant present by comparison with the molecular weight ladder.

3.3 Analysis

3.3.1 Bradford Assay for Protein Determination

The Bradford assay is a relatively quick method for the determination of protein concentration in the high molecular weight biosurfactants. The assay is based on the binding of Coomassie Brilliant Blue with protein causing a colour change that can be quantified using a spectrophotometer [33].

1. To prepare the Bradford reagent, dissolve 100 mg of Coomassie Brilliant Blue G-250 in methanol, and add 100 mL of 85% phosphoric acid. Dilute to 1 L with distilled water.
2. Prepare a series of standards (1 mL each) using bovine serum albumin (BSA) with the following concentrations: 0 (blank), 250, 500, 1,000, 1,500 and 2,000 μg/mL (*see* **Note 13**).
3. Prepare serial dilutions of unknown samples to be assayed.
4. To assay, add 1 mL of Bradford reagent and 20 μL of each of the standards into separate 1 mL plastic cuvettes (*see* **Note 14**). Prepare the unknown samples in the same manner, adding 1 mL of Bradford reagent and 20 μL into separate 1 mL plastic cuvettes.
5. Mix well and measure the absorbance at 595 nm wavelength, using the blank to zero.
6. Measure the standards and use the results to prepare a standard curve. Measure the unknown sample and use the standard curve to calculate the unknown protein concentrations.

3.3.2 Lowry Method for Protein Determination

The Lowry method [34] is an alternative method for quantifying the protein content of the purified bioemulsifiers. The method is based on the reaction between protein with cupric sulphate and tartrate in alkaline solution, which then gets reduced in the presence of Folin–Ciocalteu reagent to form a blue colour that can be measured at 750 nm using a spectrophotometer (*see* **Note 15**). This method is commonly used for protein determination of bioemulsifiers [23, 35]. This protocol is based on the modified Lowry protein assay, the reagent of which is supplied by a number of vendors and is simple to perform.

1. Prepare bovine serum albumin (BSA) standards using deionised water to obtain standards with a range of concentrations between 1,000 μg to 1 μg/mL BSA.
2. Dilute 2 N (2 M) Folin–Ciocalteu reagent to obtain a 1 M solution using deionised water (the amount required depends on the number of samples to be tested).
3. In a microtitre plate, add 40 μL of each standard and sample in triplicate to separate wells and include three blanks (deionised water).
4. Add 200 μL of Modified Lowry Reagent using a multichannel pipette to each well, and mix for 30 s using a plate mixer (can also be done by hand).
5. Seal plate and incubate for 10 min at room temperature.
6. Add 20 μL of 1 M Folin–Ciocalteu reagent prepared in step 2.
7. Measure absorbance of standards and samples using a spectrophotometer at 750 nm.
8. Subtract the average of the three blanks from all standards and samples. Prepare a standard curve and use the equation of the line to calculate the unknown samples.

3.3.3 Fatty Acid Analysis by GC-MS

Lipopeptides and high molecular weight biosurfactants vary greatly in the lipid portion; therefore, the analysis of this component provides detailed structural information for identification of fatty acid structures attached. The process involves hydrolytic cleavage of the link between the carbohydrate and peptide/protein part of the biosurfactant and the lipid portions. Subsequent derivatisation of the resulting fatty acid chains to fatty acid methyl esters (FAME), which is sufficient for analysis, or further conversion to trimethylsilyl (TMS) derivatives facilitates analysis by GC or GC-MS [36]. The method below is commonly reported for lipopeptide [21, 25] and bioemulsifiers [23, 35] using the acidic methanol approach. A further method using boron trifluoride is also given below as this method has been shown to be more efficient and less time-consuming in the generation of FAMEs and is based on the protocol AOAC 969.33 [37].

Acidic-Catalysed Methylation FAME Production

1. Take 5 mg of purified glycolipid and dry completely.
2. To this, add 0.95 mL of methanol and 0.05 mL of sulphuric acid in a sealed tube and heat at 90°C for 15 h.
3. Evaporate the solvents and extract the residue with 1 mL of hexane.
4. Wash with 1 mL of water and evaporate the hexane to obtain extract containing the fatty acid methyl esters (*see* **Note 16**).
5. GC-MS analyses can be carried out using a variety of columns such as DB-23 (Agilent) capillary column or Supelco Omegawax (Sigma-Aldrich, Dorset, UK) (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 and then ramp at 25°C/min to 175°C and then 4°C/min to 230°C and hold for 5 min (*see* **Note 17**).
7. Mass spectrometry settings; electron impact at 70 eV with scan range 50–650 Da and an injection volume of 1 μL.
8. Fatty acid methyl esters retention times increase with chain length and degree of separation.

Boron Trifluoride FAME Production

1. Weigh 50 mg of sample (*see* **Note 18**) into a screw cap tube or reaction vial.
2. Add 2 mL of 2 M sodium hydroxide in methanol (prepared by adding 8 g sodium hydroxide to 100 mL of methanol).
3. Close tube and heat at 80°C for 1 h and then allow the vial to cool.
4. Add 2 mL of boron trifluoride (25%) in methanol (can be purchased from a range of suppliers), close the tube and heat for 1 h at 80°C and then allow the vial to cool.
5. Add 5 mL of deionised water and 5 mL of hexane and shake well.
6. Allow the two phases to separate and transfer the top layer to a GC vial.
7. GC-MS analyses can be carried out using a variety of columns such as DB-23 (Agilent) capillary column or Supelco Omegawax (Sigma-Aldrich, Dorset, UK) (30 M × 0.25 mm × 0.25 μm id).
8. Analytical conditions that can be used are injector temperature 250°C, start oven program at 50°C, hold for 1 min then ramp at 25°C/min to 175°C and then 4°C/min to 230°C and hold for 5 min (*see* **Note 17**).

3.3.4 Carbohydrate Determination for High Molecular Weight Biosurfactants

The analysis of the carbohydrate portion of glycoproteins, lipopolysaccharides and other lipid-containing polysaccharide–protein complexes can be carried out using a variety of techniques. However, total characterisation of the exact structural conformation and linkage sites is extremely difficult. A two-stage approach may be adopted where the relative percentage of the carbohydrate portion of the purified biosurfactant is first determined by colorimetric assay. In the second step, the composition of the individual monosaccharides present is determined using GC-MS.

Colorimetric Assay (Phenol–Sulphuric Acid) for Carbohydrate Determination

The carbohydrate portion needs to be removed and hydrolysed into individual monosaccharides before the assay can be carried out. A number of different methods can be used to determine the carbohydrate content with the phenol–sulphuric acid method, the most commonly employed method for carbohydrate determination in bioemulsifiers [22, 23, 30].

1. Hydrolyse the purified biosurfactant product by adding 20 μg to 200 μL of 0.5 M methanolic HCl in a sealed vial (*see* **Note 19**). Heat at 65°C for 16 h.
2. Dry down using a stream of nitrogen.
3. Prepare phenol reagent by dissolving 5 mL in 95 mL of water.
4. Prepare a blank by mixing 100 μL of water to 200 μL of phenol reagent. For samples, dissolve 5 mg in 1 mL of water, take 100 μL and add 200 μL of phenol. Prepare a series of standards by dissolving glucose and 1 mg/mL. Take 20 μL, 40 μL, 60 μL, 80 μL and100 μg make up to 100 μL with water, and add 200 μL of phenol reagent to each standard [38].
5. Add 1 mL of concentrated sulphuric acid and leave solution to set for 10 min.
6. Shake vigorously and leave for 30 min.
7. Determine the absorbance at 490 nm and compare against the range of standards prepared in step 4.

GC-MS Determination of Carbohydrate Portion

After hydrolysis of the carbohydrate portion into individual monosaccharide, they can be converted to TMS derivatives for analysis by GC-MS [39]. Alternatively permethylation or peracetylation can be carried out for monosaccharide analysis.

1. Hydrolyise the purified biosurfactant product by adding 20 μg to 200 μL of 0.5 M methanolic HCl sealed vial. Heat at 65°C for 16 h.
2. Dry down using a stream of nitrogen and wash with hexane to remove nonpolar material.
3. Dissolve 10 mg of the residue with 0.1 mL of TMSI (*N*-trimethylsilylimidazole) and incubate at room temperature for 30 min.

4. Evaporate the mixture using nitrogen and dissolve in 1 mL of hexane.
5. GC-MS analyses can be carried out using a variety of columns such as DB-5 capillary column or Supelco Omegawax (Sigma-Aldrich, Dorset, UK) (30 M × 0.25 mm × 0.25 μm id).
6. Analytical conditions are injector temperature 200°C, start oven program at 50°C, hold for 3 min then ramp at 20°C per min to 170°C and then to 250°C at 6°C per min and hold for 5.7 min (*see* **Note 17**).
7. Mass spectrometry conditions; electron impact at 70 eV with scan range of m/z 50–650 and an injection volume of 1 μL for both samples and standards.
8. Identify the components by mass spectra and retention times.

3.3.5 Protein Digestion with Proteases

Using proteomic techniques, it is not always possible to obtain sufficient information to identify the amino acid sequence of the intact protein structure. This is due to several reasons, such as an inability to obtain accurate measurements on very large proteins and the fact that Edman degradation and mass spectrometry techniques are more suited to smaller peptide analysis. Therefore digestion of the protein structure into smaller peptides containing between 6 and 20 amino acids is necessary. Using individual peptides, Edman degradation and MS/MS techniques can be used to provide the information required to identify their structure and help to piece together the full protein structure. The digestion of the protein is carried out using proteases, with trypsin being the most commonly employed. Trypsin cleaves proteins at lysine and arginine residues in the C-terminal direction except where a proline residue follows. It may also be necessary to use other proteases at a later stage to help determine the order of each peptide sequence in the protein structure. For example, by using a protease such as glu-C, which cleaves at glutamate and aspartate residues, would provide a different set of cleaved peptides. Investigation of these peptides would provide information allowing overlapping portions of the sequence to be identified. The digestion described here is based on in-solution tryptic digestion [40], however in-gel digestion is also commonly used. Identification of the protein structure present in bioemulsifier has not been reported to date, probably due to the difficult nature, but the method below along with de novo sequencing could provide new insights into the chemical structures of protein-containing bioemulsifiers.

1. Prepare ammonium bicarbonate buffer by adding 400 mg to 100 mL of ultrapure water.
2. Prepare sample buffer of mixture of acetonitrile/water (*see* **Note 20**) in ammonium bicarbonate buffer containing 50 mM Tris–HCl (pH 7.6) and 10 mM $CaCl_2$.

3. To make reduction solution, add 16 mg of dithiothreitol (DTT) in 500 μL ultrapure water to give concentration of approximately 200 mM. The alkylation solution is prepared by adding 9 mg of iodoacetamide to 500 μL of ultrapure water to give a concentration of 100 mM.
4. Prepare 10% formic acid solution.
5. Dissolve the purified protein band obtained from gel electrophoresis technique described in Sect. 3.2.6 in 40 μL of sample buffer made in step 2.
6. For reduction, add 4 μL of 200 mM DTT prepared in step 3 and incubate at 37°C for 1 h.
7. For alkylation, add 4 μL of iodoacetamide solution prepared in step 3, and incubate for 15 min in the dark at room temperature.
8. To digest the protein, add trypsin (*see* **Note 21**) in a ratio of trypsin/protein 1/5 and incubate for 2–4 h at 37°C.
9. To stop the digestion, add formic acid to a final concentration of 5% (v/v).
10. The sample can then be submitted for separation using HPLC as described in Sect. 3.2.2 to obtain individual peptides for MS/MS analysis. Alternatively the mixture can be purified to remove the buffers and used for MALDI-TOF analysis and capillary chromatography with ion trap/QTOF MS/MS.
11. The sample can be lyophilised and stored at −80°C.

3.3.6 MALDI-TOF Mass Spectrometry

MALDI is a soft ionisation mass spectrometry technique that allows the identification of intact compounds. Samples to be analysed are mixed with a matrix and dried on a platform, onto which a laser is fired with various degrees of energy forming gaseous ions, which can then be separated in a time-of-flight analyser (TOF) and detected. MALDI can be used to determine the full molecular mass of high molecular weight biosurfactants and lipopeptide biosurfactants that have been purified. It is also possible to analyse the individual peptide components obtained from the protease digestions and use the information obtained to search a database in a process known as peptide mass fingerprinting or peptide mass mapping. If the high molecular mass biosurfactant has been previously characterised, the ions observed for protease digestion would be specific for this biosurfactant and therefore sufficient for identification. MALDI has been employed for bioemulsifiers [22] and routinely for lipopeptide analysis [10, 41, 42].

1. Prepare the matrix for peptide analysis, α-cyano-4-hydroxy cinnamic acid (αCHCA), which should be used for compounds up to 10,000 Da. Prepare a 10 mg/mL solution of αCHCA in acetonitrile/ultrapure water/trifluoroacetic acid (50:50:0.1) and vortex until dissolved.

2. Prepare matrix for high molecular weight biosurfactants (>10,000) as for step one but using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid).
3. Dissolve sample in methanol, mix 1 μL with 1 μL of matrix and apply to sample plate. Allow plate to dry at room temperature
4. Insert plate into instrument, move plate to sample position and fire the laser.
5. If the signal is poor, increase laser strength or move the laser to a different position on the well (*see* **Note 22**).
6. To improve signal, remove possible salts that maybe present by washing the sample on plate with 2 μL of cold ultrapure water containing 0.1% TFA.

3.3.7 Edman Degradation for Amino Acid Sequence

Edman degradation is the classic technique for sequencing peptides using chemical methods and may be applied to peptides and proteins where the N-terminus has not been modified by, for example, pyroglutamic acid or acetylation [43]. The method provides an assignment for each residue in the peptide, unlike amino acid analysis which only provides an indication of the ratio of AAs in the peptide. Edman experiments take place in an oxygen-free environment and involve the modification of the N-terminal residue with phenyl isothiocyanate to provide a cleaved phenylthiohydantoin (PTH) amino acid. All of the chemical processes take place on automated sequencers and are followed by a chromatographic step where the retention time of the cleaved PTH amino acid is compared with the retention times of a series of PTH-modified amino acid standards to ascertain its identity. The method is relatively slow taking approximately 45 min for each residue; however, the amino acid assignment is called with a high degree of confidence and less subject to interpretation, as can be the case with MS/MS methods. Of particular relevance is the differentiation between isobaric leucine and isoleucine which is not possible with low energy collision MS/MS but readily achievable using Edman degradation. The quality of sequence information obtained by the Edman method is subject to the amount of starting material and its purity (*see* **Note 23**). For successful sequencing to take place, peptides and proteins must be purified to near homogeneity by chromatographic methods to prevent mixed sequencing during Edman experiments. Lipopeptides need to be in the open ring form for this type of analysis, which is carried out using mild alkaline hydrolysis.

1. Purify the protein or peptide of interest using chromatographic methods, described in Sect. 3.2.2, removing all the non-volatile salts and buffers that may cause unwanted side reactions.
2. Take a small sample of dried purified cyclic lipopeptides, dissolve in 1 M NaOH and keep at room temperature for 12 h.

3. Neutralise the mixture with HCl and extract the linear form of the biosurfactant by extracting with chloroform.
4. Remove the salts that were used for opening of the cyclic peptide ring using HPLC as per Sect. 3.2.2.
5. Apply the sample to the instrument using manufacturer's instructions and carry out sequencing. Continue until no further sequence information is obtained.

3.3.8 Capillary Chromatography with Ion Trap/QTOF MS/MS for De Novo Sequencing

The use of QTOF MS/MS and other high-resolution mass spectrometers such as orbitraps and FT-ICR instruments allow de novo sequencing of peptides where the mass accuracy capability of the instrument facilitates amino acid assignment of the peptide without recourse to a database [44]. The method is useful where the protein or peptide of interest is deemed novel due to unsuccessful sequencing attempts by database searching with MS/MS datasets. Protease digestion of large proteins must first occur as the technique is most successful for peptides less than 2,000 Da. Unlike Edman degradation, MS/MS experiments do not require the peptide to be purified to homogeneity as the mass analyser may be used to select only the ions of interest. The analysis can be carried out using a direct infusion method or by capillary liquid chromatography, nano LC, UPLC or HPLC separation of individual peptides before they enter the mass spectrometer. Following fragmentation of the peptide, various proprietary software tools are available to process the raw data and analyse the sequence assigned. Frequently this is used in conjunction with BLAST searching to determine if homology exists with existing precursor structures. Often low mass resolution instruments, in particular ion trap technology, are used to obtain MS/MS data, which may be analysed with appropriate algorithms to provide sequence assignment based on database searching. While very successful, the resolution of the instrument does not allow for de novo sequencing. Advantages of the method include fast analysis times and the small sample volume required. Disadvantages of the method are that algorithms are used to assign a peptide sequence to product ion spectra and a certain level of expertise must be gained to critically analyse the data produced. Often, de novo sequencing is used to identify sequence tags within the peptide structure rather than the complete sequence. These tags may then be used to design primers for cDNA cloning studies that may further corroborate the mass spectrometric sequencing data. As with all MS equipment, the experimental technique will vary greatly depending on the manufacturer of the instrument and software; therefore, only specific details on sample preparation and basic operation procedures are described below, and for specific details the software manuals should be consulted.

1. Following trypsin digestion for protein-containing biosurfactants and after chromatographic purification of the peptides or lipopeptides (Sects. 3.3.5 and 3.2.2).
2. Prepare samples for analysis by dissolving in acetonitrile/water mixture.
3. Samples may be analysed by capillary chromatography mass spectrometry or by introducing the sample directly into the instrument using a spray emitter, according to the manufacturer's instructions.
4. Connect appropriate capillary column.
5. Capillary liquid chromatography conditions, starting at 100% mobile phase A (HPLC water containing 0.1% formic acid) changing to 100% mobile phase B (acetonitrile/water/formic acid 80:20:0.1) over 1 h.
6. Set flow rate at 0.3 μL/min with an injection volume of 5 μL.
7. Analyse data according to software for de novo sequencing.

3.3.9 HPLC with QTOF MS/MS for De Novo Sequencing

While capillary LC is routinely used for mass spectrometric analysis and for de novo sequencing on QTOF mass spectrometers, it is generally used for complex peptide mixtures and when only small quantities of the peptides are available. Lipopeptides biosurfactants can be obtained in reasonable quantities, and their relatively limited complexity permits the use of standard HPLC (using narrow-bore HPLC columns) separation prior to analysis using QTOF instrumentation for de novo sequencing. The method detailed below uses a narrow-bore HPLC column resulting in greater resolution, speeds off analysis and could be applied to other MS instruments. A range of different methods have been used for lipopeptide MS sequencing with quadrupole ion trap MS [18, 38], direct infusion QTOF MS/MS [25, 29] and LC-QTOF MS/MS [28, 41] methods reported. The method below was carried out using a Waters Alliance HPLC coupled to a Waters QTOF Premier mass spectrometer [45, 46].

1. Connect a Waters Atlantis reverse phase C_{18}, 2.1 × 100mm, 2.7 μm particle size column (or equivalent from another supplier) to the HPLC, and set the column oven to 40°C.
2. Set the following parameters on the mass spectrometer tune method: positive mode ionisation, source temperature 120°C, desolvation temperature 300°C, desolvation gas 800 L/h and cone gas 50 L/h. The capillary voltage should be set to 3.0 kV and cone voltage to 35 V.
3. Set up mass spectrometry method performing collision-induced dissociation (CID) of the analytes in MS^e mode with argon by ramping up the collision energy from 20 eV to 30 eV. Set the mass range of m/z 100–1,400.

4. Dissolve samples at concentration of 0.1 mg/mL in methanol.
5. Prepare mobile phase A of HPLC water containing 0.05% formic acid and mobile phase B consists of HPLC acetonitrile containing 0.05% formic acid.
6. Set up inlet method with isocratic elution using the following method at a flow rate of 0.2 mL/min: 60:40 (mobile phase A–mobile phase B).
7. Use an injection volume of 2 μL.
8. Set up sample list using the mass lynx Waters software and run analysis.
9. Process data according to software for de novo sequencing.

3.3.10 NMR Analysis of Lipopeptides

The previously reported methodologies allow the identification of lipopeptides to a reasonable degree of certainly. Generally the fatty acid portion and the peptide section are analysed separately. However, NMR can be used. NMR confirms the identification of the structure of both the peptide portion and the fatty acid while providing conformation of the position of linkages between the peptide and the fatty acid chain. The lipopeptides should be dissolved in deuterated 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. This technique is regularly used to determine the fine structure of the intact lipopeptide [28, 29, 38, 46].

4 Notes

1. A small amount of formic acid (1%) can also be added for extraction.
2. Higher saturation levels may be required for precipitation of some proteins. Refer to literature of the specific biopolymer under investigation.
3. Washing the pellet with hexane can be carried out, if required, to remove nonpolar lipids.
4. Other TLC solvents could be used for analysis. This system is for general lipopeptide use; however, more specific systems can be found in the literature for particular strains.
5. Using a C18 column provides a good starting point for analysis and generally should result in a good separation; a large variety of other columns could also be used.
6. Generally a number of trail runs should be carried out to optimise the column conditions and the run time required. Time given is just a rough guide but can be adjusted up or down depending on the sample.

7. GPC can also be carried out using an Evaporative Light Scattering Detector (ELSD), but the mobile phase should be changed to contain only volatile buffer components such as ammonium acetate/formate.
8. Only a relatively simple mixture is present. When complex mixtures are being studied, 2D gels are more appropriate.
9. The % gel depends on the size of proteins. 10% is suitable for the range 20–300 kDa approximately.
10. The majority of these buffers can be purchased as ready-made solutions.
11. Dithiothreitol (DTT) can also be used.
12. Other stains can also be used.
13. The method detailed is for a standard assay; scaling down can be carried out for a microassay. For microassay (1–10 μg of protein), use standard concentrations of 1, 5, 7.5, 10, 15 and 20 μg of protein.
14. For microassay preparation, use 1 mL of Bradford reagent and 100 μL of standard or sample.
15. As with all colorimetric assays, the presence of some impurities may influence the results.
16. The FAME can be analysed by GC or GC-MS at this stage; however, further derivatisation to TMS derivatives can in some cases provide better sensitivity for analysis.
17. 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.
18. This procedure can be scaled down tenfold if starting materials are low.
19. TFA can also be used at stage instead of sulphuric acid, using 4 m TFA at 100°C for 4 h [23].
20. Sample buffer should contain at maximum 80% acetonitrile; the value should depend on the protein solubility and should be checked beforehand.
21. It may be necessary to use other proteases in order to obtain further information about the order of the sequences obtained.
22. Signal is severely influenced by the presence of salts and other impurities.
23. If no sequence information is obtained during the first three or four cycles, this may indicate that the N-terminus is blocked and may require treatment with an enzyme such as PyroGlu aminopeptidase to remove the modification. Sequencing may then commence at residue two of the truncated peptide.

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Protocols for the Detection and Chemical Characterisation of Microbial Glycolipids

Thomas J.P. Smyth, Michelle Rudden, Konstantina Tsaousi, Roger Marchant, and Ibrahim M. Banat

Abstract

Microbial-produced biosurfactant glycolipids consist of four major groups: rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids. Extensive research has been carried out on the production and analysis of rhamnolipids and sophorolipids, with significantly less research publications in the area of trehalose lipids and mannosylerythritol lipids. When studying these microbial glycolipids, the ability to isolate, purify and characterise the structures produced by fermentation process is extremely important. Chemical and structure information obtained from the glycolipid products provides invaluable information on the different conditions, such as microbial strain type, carbon sources and temperature, which influence production of glycolipids. The information from quantification and chemical analysis of these glycolipids assists in the optimisation of production yields and assembly of glycolipids with different structural characteristics. Armed with this information, the manufacture of glycolipids can be manipulated with different fermentation approaches to tailor the production of different types of glycolipid depending on the biological activity required. The experimental techniques used to isolate, purify and analyse glycolipids are extremely varied with each method having a range of pros and cons. These methods range from simplistic methods such as colorimetric assays that provide rough indication of production yields in an inexpensive manner, extending to complex mass spectral techniques, which necessitate sophisticated instrumentation and scientific expertise. This chapter therefore details information and protocols that are essential for the detection and chemical characterisation of glycolipids. Since there is an abundance of methods available, only the most commonly described techniques are detailed in this chapter.

Keywords: Bioemulsifiers, Biosurfactants, Glycolipids, Mannosylerythritol lipids, Quantification and chemical analysis, Rhamnolipids, Sophorolipids, Trehalose lipids

1 Introduction

Biosurfactants are amphiphilic compounds with both hydrophilic and hydrophobic moieties (mostly hydrocarbons) able to display a variety of surface activities that, among other roles, help in producing microorganisms to solubilise hydrophobic substrates [1]. In recent years, biosurfactants have been investigated as potential replacements for synthetic surfactants and may have many potential

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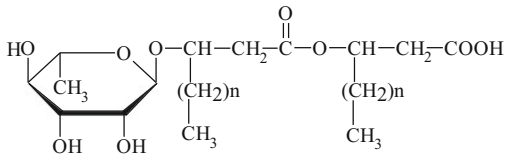
industrial and environmental applications [2, 3]. Biosurfactants represent a family of structurally diverse molecules with high and low molecular weights. This chapter will concentrate on the low molecular weight biosurfactants and will focus mainly on glycolipids consisting of carbohydrates joined to fatty acids or hydroxy fatty acid chains. There are four major groups of microbial glycolipids: rhamnolipids, sophorolipids, trehalose lipids and mannosylerythritol lipids (Fig. 1).

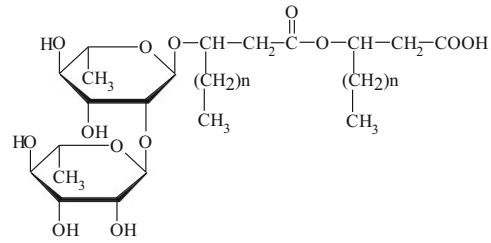
Rhamnolipids, produced predominantly by *Pseudomonas aeruginosa*, consist of either one or two rhamnose units connected to one or two β-hydroxy fatty acid chains with a length of 8 to 12 carbons but with decanoic acid (C_{10}) being the most abundant [4]. Sophorolipids consist of two major groups, the acidic sophorolipids comprising a disaccharide, sophorose, linked to the subterminal or terminal carbon of the fatty acid chain and the lactonic sophorolipids where the carboxylic acid portion of the fatty acid is joined to C-4 position of the disaccharide unit [5]. Sophorolipids are most commonly produced by *Starmerella* (*Candida*) *bombicola* and *Candida apicola*, along with a number of other species of this clade. Sophorolipids are predominantly synthesised with C_{18} as the most abundant hydroxy fatty acid terminally linked to the sophorose; however, a large number of structural variations has been reported [6, 7]. Trehalose lipids are made up of a disaccharide, trehalose, linked by an ester bond to α-branched β-hydroxy fatty acids [8]. The α-branched β-hydroxy fatty acids are connected at the C6 and C6′ of the carbohydrate structure in the case of the trehalose dimycolates and at C6 for the monomycolates; other structure types have also been reported [8]. The production of trehalose lipids is associated with most species of *Mycobacterium*, *Rhodococcus* and *Corynebacterium* [9]. 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 [10]. 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 employed for glycolipid analysis depend on the amount of detailed information required; however, the experimental procedure should rely on the following key steps: (1) extraction of glycolipids from the culture medium, (2) glycolipid detection and rough quantification, (3) purification and separation of the crude product (4) and finally structural analysis, through a combination of LC, MS and NMR techniques.

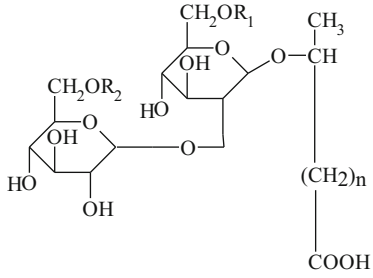
A thorough identification as well as quantification of a glycolipid molecule would need a combined use of some or all of these resources together to overcome the further difficulties caused by the presence of various co-extracted impurities including excess



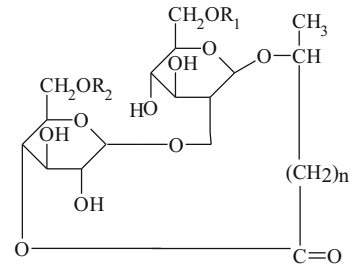
Monorhamnolipids



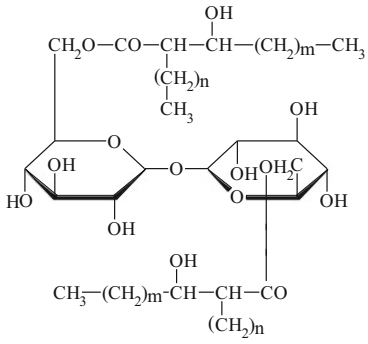
Dirhamnolipid



Acidic Sophorolipid

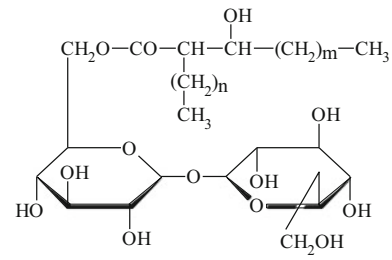


Lactonic Sophorolipid



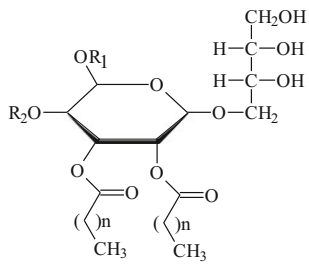
m+n =27 TO 31

Trehalose dimycolates



m+n =27 TO 31

Trehalose monomycolates



Mannosylerythritol lipids

Fig. 1 The basic structures of each of the four major groups of microbial glycolipids. *n* represents the length of the lipid chain, while R can be either H or acetyl group

carbon source and the range of glycolipid compounds and congeners produced by the selected microorganism.

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. Table 1 provides a simple insight into the specific advantages and disadvantages of each method. Further information on each procedure for analysis and information regarding interpretation of results can be found in the references listed.

2 Materials

1. Extraction: ethyl acetate (for rhamnolipids (RL), sophorolipids (SL), mannosylerythritol lipids (MEL)); chloroform and methanol (for trehalose lipids (TL)); magnesium sulphate (for RL, SL, MEL); concentrated HCl (RL, TL).
2. Colorimetric detection: specific for anthrone assay (anthrone, absolute ethanol, concentrated sulphuric acid and distilled water); specific for orcinol assay (orcinol, concentrated sulphuric acid, distilled water and ice).
3. TLC: TLC chamber tank, TLC plates generally silica gel 60 or equivalent, spray reagent bottle, methanol, chloroform; acetic acid (RL); distilled water (SL, TL); 7N ammonium hydroxide; p-anisaldehyde 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), 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: chloroform, methanol, sulphuric acid, distilled water (for RL, TL, MEL); methanol, 1% sulphuric acid, toluene, cyclohexane, sodium chloride (for SL).
6. HPLC-UV: all for rhamnolipid, HPLC utilising UV detection with C_{18} column, water, acetonitrile, 2-bromoaceto-phenone, triethylamine, water bath, 0.22 μm syringe filters, 3.3 mM H_3PO_4.
7. HPLC-ELSD: HPLC grade acetonitrile, HPLC water (all for RL, SL, TL, MEL); trifluoroacetic acid (for RL).

Table 1
Comparison of the most commonly employed analytical methods for glycolipid analysis

Analytical method	Advantage	Disadvantage
Indirect analysis		
Surface/ interfacial tension	Quick, easy to use	Qualitative analysis, no structural information obtained
Orcinol/ anthrone assay	Cheap and simple	Inaccurate, interferences from co-extracted analytes particularly residual carbon sources
Chromatographic separation		
TLC	Simple, inexpensive, separates into distinct glycolipid groups	Not suitable for quantification, cannot identify mixtures of congeners, subject to interferences from other impurities and excess lipid carbon sources
HPLC	Speed, highly reproducible, accurate, suitable for scale up, i.e. semi-prep, high throughput	Co-elution of unwanted compounds, difficult to fully resolve complex samples
UPLC	Increased sensitivity and reduced run time due to column particle size, less solvent consumption	High maintenance, columns expensive, high pressure
Structural identification		
ESI-MS^n	Rapid, excellent for profiling and for checking purity, can be easily coupled to LC, sensitive, fragmentation patterns for identification of structural isomers	Glycolipid may be affected by ionisation efficiency, ion suppression from co-extracted material such as salts and lipids, subject to in-source fragmentation
GC-MS	For nonpolar volatile compounds, suitable for determination of lipid chains after cleavage from glycolipid, provides exact chain length and position of double bonds if present	Requires derivatisation, not fully quantitative without standard curve calibration, provides information of lipid chains present but not in relation to the full glycolipid structure particularly for glycolipids containing more than one lipid chain (relationship between the 3-hydroxyfatty acids in the di-lipid portion of the RLs is lost, along with the relationship between the di-lipids and their substitution with one (or two) rhamnose moieties)
HPLC-MSMS	Identify glycolipids without the need for complete separation, CID fragmentation identification of isomers, analyses of a wide range of glycolipids,	Needs external standard curve calibration for quantification, matrix effects, purity, concentration can also influence method, expensive and requires technical expertise to operate

(continued)

Table 1
(continued)

Analytical method	Advantage	Disadvantage
HPLC-ELSD	Universal detection, no need to derivatise analyte, no interference from solvents, cheaper than MS detectors	Low sensitivity for low molecular weight compounds, cannot identify isomers, need external standard curve calibration for accurate identification and quantification
HPLC-UV	Relatively inexpensive, can be quantitative	Requires derivatisation to add a chromophoric group into the glycolipid structures, difficult to obtain full derivatisation
MALDI-TOF	High molecular wt. range (400–350,000 Da). More salt/buffer tolerant, better for complex mixtures, samples do not need to be volatile, quick, no in-source fragmentation	Poor reproducibility (hot spots), difficult to identify isomer, impurities might not be observed if they cannot be ionised using this approach
UPLC-MSMS	Specific, high throughput, speed, sensitivity and high resolution, quantify trace amounts of analytes, MRM mode results in reduced matrix effects	Expensive, sensitive – requires high/frequent maintenance, in MRM mode only shows targeted glycolipids, requires significant technical expertise to operate
NMR	Non-destructive, detailed structural information can be obtained, stereochemistry, bond distances and angles can be observed	Difficult to analyse complex mixtures and large molecules, expensive, requires significant technical expertise to operate and interpret data

8. ESI-MS: 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 ionisation, matrix (α-cyano-4-hydroxy cinnamic acid (αCHCA)), acetonitrile and ultrapure water.
11. NMR: NMR equipment, deuterated chloroform and NMR tubes.

3 Experimental Approach

3.1 Colorimetric Analysis

Colorimetric methods can be used to determine the presence of biosurfactants in either the culture medium or the extract. These assays are used to detect the sugar moieties of the glycolipids with the anthrone [11] or orcinol assay being the most commonly used.

The major advantage of these methods is that they can be carried out directly on the culture medium after cell removal 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.

3.1.1 Anthrone Assay

The anthrone assay can be used to detect and roughly quantify the amount of glycolipid present in the culture broth [11]. In this method, sugars react with the anthrone reagents in the presence of sulphuric acid to produce a blue-green colour. This assay is still commonly employed [12] and can be used for all types of glycolipid biosurfactants by detecting 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% sulphuric acid.
2. Prepare a standard curve using a pure sample of the glycolipid being investigated at a range of concentrations between 0 and 50 μg/ml in water. Similarly, a curve can be generated using a standard of rhamnose with the concentration of rhamnolipid being calculated by multiplying the amount of rhamnose by a conversion factor of 3.4 [13].
3. For samples, add 200 μl of cell-free supernatant and 1,000 μl of anthrone reagent.
4. Take 200 μl of each standard and add 1,000 μl of anthrone reagent.
5. Heat in boiling water for 9 min.
6. Cool and measure absorbance at 625 nm.
7. Prepare a standard curve, and use the equation of the line to calculate the concentration of glycolipid present in samples.

3.1.2 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-coloured dye whose absorbance can be measured and used to roughly quantify the glycolipid concentration in the sample [14, 15].

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. Prepare a series of standards using purified glycolipid at concentrations of 0–50 μg/ml in water (*see* **Note 1**). Similarly, a curve can be generated using a standard of rhamnose with the concentration of rhamnolipid being calculated by multiplying the amount of rhamnose by a conversion factor of 3.4 [13].

3. To 100 µl of cell-free supernatant (or standard), add 900 µl of orcinol reagent and incubate at 80°C for 30 min. Allow mixture to cool at room temperature.
4. Measure the absorbance at 421 nm and calculate results based on the standard curve.

3.2 Extraction of Glycolipids

After completion of the fermentation process, microbial cells should be removed immediately. At this stage, a crude extract free from the aqueous culture medium and microbial cells should be obtained as the first step. Although a variety of methods are available, the most commonly used is solvent extraction. The methods reported below are the most commonly used for each of the different glycolipid biosurfactant groups.

3.2.1 Rhamnolipids

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 (*see* **Note 2**).
3. Centrifuge at 20,000 × *g* at 4°C for 20 min to obtain precipitate [16].
4. Crude rhamnolipid at this stage contains moisture and if required dry using a freeze dryer or vacuum oven.

Solvent Extraction

This extraction technique is used in combination with acid precipitation for more efficient yields and represent the most commonly utilised approach for extraction of rhamnolipids [12, 17, 18]. Solvent extraction is particularly important when limited precipitation is observed when acidified which usually correlates to low yields of rhamnolipid in the fermentation process.

1. Remove cells by centrifuging at 13,000 × *g* for 15 min (*see* **Note 3**).
2. Acidify by addition of concentrated HCl to pH 3.0 and transfer to a separating funnel.
3. Extract three times with an equal volume of ethyl acetate (*see* **Note 4**), shaking each time, and allow the two layers to fully separate.
4. Transfer bottom aqueous layer and the top ethyl acetate layer to separate flasks. Re-extract the aqueous portion twice more or until no further colour persists in the ethyl acetate layer.
5. Add 0.5 g of magnesium sulphate per 100 ml of ethyl acetate portion to remove the traces of water present and filter.
6. Rotary evaporate to yield a brown gum extract. Alternatively remove ethyl acetate using a nitrogen dryer.

3.2.2 Sophorolipids

Solvent extraction is carried out in similar manner to rhamnolipids, but without removal of cells or acidification. When high yields of sophorolipids are produced by the fermentation process (>4 g/l), the procedure becomes much easier as sophorolipids are denser than water and sediment at the end of the process [5]. The honey-like sophorolipid material at the bottom can be removed and washed several times with water; alternatively, this process can be speeded up by centrifuging to obtain sophorolipids free from the supernatant [5].

1. Remove cells by centrifuging at 13,000 × g for 15 min (*see* **Note 5**).
2. Extract three times with an equal volume of ethyl acetate (*see* **Note 4**) in a separating funnel, shaking each time, and allow the two layers to fully separate.
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 colour persists in the ethyl acetate layer.
4. Add 0.5 g of magnesium sulphate per 100 ml of ethyl acetate portion to remove the traces of water present and filter.
5. Rotary evaporate to yield a brown gum extract. Alternatively remove ethyl acetate using a nitrogen dryer.
6. Remove excess lipidic carbon source, often co-extracted with ethyl acetate, if present by washing three times with hexane.

3.2.3 Trehalose Lipids

Solvent extraction is carried out in similar manner to sophorolipids, using the whole culture broth and without acidification [19].

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

3.2.4 Mannosylerythritol Lipids (MELs)

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

1. Remove cells by centrifuging at 13,000 × *g* for 15 min (*see* **Note 3**).
2. Extract three times with an equal volume of ethyl acetate (*see* **Note 4**) in a separating funnel , shaking each time, and allow the two layers to fully separate.
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 colour persists in the ethyl acetate layer.

4. Add 0.5 g of magnesium sulphate per 100 ml of ethyl acetate portion to remove the traces of water present and filter.
5. Rotary evaporate to yield a brown gum extract. Alternatively remove ethyl acetate using a nitrogen dryer.

3.3 Thin Layer Chromatography Detection/Purification

Thin layer chromatography (TLC) is a simple method allowing identification of the presence of glycolipids. It can also provide preliminary information on structural types of glycolipids present in crude extracts, while at the same reveal the presence of purities in crude extracts. TLC detection should be carried out before purification procedures to determine the presence of glycolipids and to determine purity after purification steps. Preparative TLC can also be carried out using the methods detailed below. However, during the detection step, the majority of the plate should be covered to protect the glycolipids, and only the edge of the plate should be sprayed to determine the position of the respective glycolipid groups. In preparative scale TLC, the individual bands can be scrapped and re-extracted to obtain small quantities of each of the different glycolipid structural groups.

3.3.1 Rhamnolipid TLC Analysis

TLC is regularly used for detection and composition of rhamnolipids in culture broth extracts [18, 20]. Under normal phase TLC conditions, mono-rhamnolipids and di-rhamnolipids are separated into two bands [21]. 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) [22].
4. When developed (solvent front is roughly 1 cm from top of plate), remove plate and allow to air-dry in a fume cupboard.
5. Prepare anthrone reagent by mixing 63 ml of sulphuric acid, 25 ml of water and 0.125 g of anthrone, under ice conditions.
6. Spray the plate evenly with anthrone reagent using an atomiser or TLC sprayer, and place in an oven at 110°C for 20 min.
7. On visualisation, the spot (blue-green colour) nearer the point of origin corresponds to the di-rhamnolipids, while the spot further from the point of origin represents the mono-rhamnolipids. RF values can vary due to different plate dimensions and sample concentrations but generally are in the range 0.3–0.4 for di-rhamnolipid and 0.75–0.85 for mono-rhamnolipids [23]. On a preparative scale, the bands can be scraped and extracted to yield purified rhamnolipids.

3.3.2 Sophorolipid TLC Analysis

TLC analysis of sophorolipids results in a greater number of TLC spots corresponding to the different types of sophorolipids and is regularly reported [24]. Generally the major sophorolipids spots occur in the following order (from bottom of the plate to the top): acidic with no acetyl groups, acidic with one acetyl group, acidic with two acetyl groups, lactonic with no acetyl groups, lactonic with one acetyl group and finally lactonic with two acetyl groups.

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 sophorolipids should be applied as standards for comparison.
3. When dry, develop in a TLC tank pre-saturated with the solvent system of chloroform-methanol-water (6.5:1.5:0.2, v/v/v) [7].
4. When developed (solvent front is roughly 1cm from top of plate), remove plate and allow to air-dry in a fume cupboard.
5. To detect the presence of sophorolipid, spray with anisaldehyde reagent. This can be prepared by mixing 100 ml acetic acid, 2 ml of sulphuric acid and 1ml of *p*-anisaldehyde.
6. Compare TLC spots obtained with published data [7]. Acidic sophorolipids appear near the point of origin, while the lactonic forms appear above. Care should be taken to avoid confusion of spots near the solvent front when excess lipid carbon sources are still present in the crude sophorolipid extract.

3.3.3 Trehalose Lipids TLC Analysis

TLC is carried out according to the procedure for sophorolipids at Sect. 3.3.2. Results can be compared with published data with trehalose monomycolates appearing near the point of origin with trehalose dimycolates slightly above [25, 26]. Trehalose lipids will appear green using *p*-anisaldehyde. Other spots are likely to be detected corresponding to other components of the trehalose lipid extract.

3.3.4 Mannosylerythritol Lipid TLC Analysis

Detection of MELs can be carried out in a similar manner to that used for rhamnolipids with the following modifications [27]. TLC is commonly employed to show the presence of each of the major groups of MELS [28].

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. Use a previously purified sample of MELs as a standard.
3. Develop plate in solvent system of chloroform-methanol-7N ammonium hydroxide (6.5:1.5:0.2, v/v/v).
4. When developed (solvent front is roughly 1cm from top of plate), remove plate and allow to air-dry in a fume cupboard.

5. Prepare anthrone reagent by mixing 63 ml of sulphuric acid, 25 ml of water and 0.125 g of anthrone, under ice conditions.
6. Spray the plate evenly with anthrone reagent using an atomiser or TLC sprayer, and place in an oven at 110°C for 20 min.
7. Compare results with published data [29]. Using this procedure, separation occurs from the point of origin to the solvent front in the order MEL D to MEL A.

3.4 Purification by Column Chromatography

During the extraction procedures, a number of impurities are often co-extracted including excess carbon sources particularly when oils and alkanes are used for glycolipid production. Similarly, other impurities such as excess glycerol and partially constructed glycolipid products can also be present. The type of impurity and their quantity can have negative effects on the subsequent glycolipid chemical characterisation. While traces of sugar-based feeding sources have limited impact on subsequent mass spectrometry analysis, the presence of excess lipidic material can be detrimental and in many cases completely inhibit structural characterisation. Furthermore, it is often necessary to study the bioactive or surface active properties of the various types of biosurfactant present in an extract by separating the different structural components. Column chromatography is a relatively inexpensive method regularly used to purify glycolipids. Using this technique, milligram to kilogram quantities of glycolipids can be obtained free from impurities and at the same time result in the separation of structural groups of glycolipids for further analysis. These column chromatographic techniques can range from simple open glass columns to automated flash chromatography systems. Similarly, using the same gradient conditions detailed below, solid phase extraction (SPE) cartridges are a cheaper alternative method.

3.4.1 Rhamnolipids Column Chromatography

Using this technique excess carbon sources such as fatty acids and other impurities that are co-extracted with the glycolipids can be removed [30]. This method is regularly used to obtain pure samples of both mono-rhamnolipids and di-rhamnolipids for further investigation [31, 32]. The use of chloroform results in the silica gel turning translucent allowing easy visualisation of both the mono- and di-rhamnolipid band.

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 the majority of solvent is absorbed; dry using rotary evaporation. Pour into column and add another layer of sand.
5. Wash column with chloroform until no colour persists to remove neutral lipids and nonpolar pigments.
6. Change solvent conditions to chloroform-methanol (97:3, v/v) followed by 95:5, v/v and continue elution until the observed band corresponding to mono-rhamnolipids is eluted, collecting the elute solvent prior to band eluting and for several minutes after.
7. Elute the di-rhamnolipids with chloroform-methanol (50:50, v/v), collecting just before the band elutes and for a couple of minutes after.
8. Dry down each fraction using rotary evaporation or nitrogen concentrators and TLC to confirm purity of rhamnolipids.

Flash Chromatography

Fractionation can also be carried out using an automated flash chromatography system such as a Varian IntelliFlash 310 Flash Chromatography (Analogix Semiconductor Inc., California, USA) system using an Agilent SuperFlash™ column. When using these systems, the flow rate and sample loading capacity should be used according to the manufacturer. For example, when using a SF40–240 g normal phase silica column (40.6 mm × 37.9 cm, 50 μm), a flow rate of 60 ml/min should be used throughout.

1. Condition the column with three column volumes of hexane.
2. Add 4 g of crude extracted rhamnolipid dissolved in chloroform and injected onto the column.
3. Wash column with three column volumes of hexane followed by washing with chloroform to remove lipid material until no further colour was eluted for each solvent.
4. Elute mono-rhamnolipids with 97:3 (chloroform-methanol) (the mono-rhamnolipid band can be collected based on visualisation of the column and UV detection).
5. Elute di-rhamnolipid with 50:50 (chloroform-methanol) with collection of the visualisation of the rhamnolipid band.
6. Dry both fractions using rotary evaporation.

3.4.2 Sophorolipids

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 nonpolar lactonic structural group. Therefore, while the separation into relatively pure acidic and lactonic groups can be quite easily carried out, purification of each structural group within the acidic or lactonic groups is difficult to be fully achieved [33]. Several methods are available using column chromatography [34, 35], but each requires profiling of fractions by TLC or mass spectrometry to determine separation of the different structural groups. Other approaches utilising medium pressure column chromatography (MPLC [7, 36] and flash chromatography, which can be used with the same conditions as detailed below) can also be applied to sophorolipid purification. Crystallisation is also a useful approach to isolate lactonic sophorolipid but requires a high percentage of lactonic sophorolipid in the crude mixture to be successful [37].

The method should be carried out according to section "Column Chromatography" 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 (*see* **Note** 7).
2. For step 8, depending on the quantity of extract and size of column, equal volume fractions should be taken and analysed by TLC and combined based on their profiles. Comparison with the sophorolipid TLC method [7] can help to tentatively identify the structural groups of sophorolipids. Similarly, samples can be quickly analysed by mass spectrometry to obtain accurate fraction composition.

3.4.3 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 the 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 [25]. Modifications of the method below can also be found in the literature [26].

Initial Column Chromatography 1

1. To remove excess hydrocarbon substrates, set up column according to section "Column Chromatography" steps 1–5 with the following modifications.
2. At step two, use silica gel 60 with mesh size of 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. [25] to detect each component.

1. Carry out purification of trehalose lipid according to section "Column Chromatography" 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).

3.4.4 Mannosylerythritol Lipids

MELs can be separated using silica gel column chromatography [38] and are reported frequently with slight variations of the original method reported here [28, 39, 40].

1. Perform separation according to procedure in section "Column Chromatography" 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 colour persists to remove neutral lipids and nonpolar 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.

3.5 Chemical Analysis

After extraction and partial purification of glycolipids have been completed, they can be subjected to chemical characterisation. Chemical analysis is required to both quantify and/or investigate structural features of these sometimes complex glycolipid groups. Numerous techniques are currently used to analyse glycolipids with the majority of methods analysing the intact glycolipid structure. However, analysis can also be completed on its constituent sugar and lipidic moieties after cleavage of the link between these two components. Among these techniques, mass spectrometry offers the greatest amount of information with regard to purity and structural conformation.

3.5.1 Lipid Analysis

The majority of the structural variation that arises in glycolipid biosurfactants occurs in the lipid portion; therefore, analysis of this part of the structure provides detailed information that permits the full identification of glycolipid structures. In the most part, the variation is usually based on the chain length and the level of unsaturation, which can easily be obtained using LC-MS-based techniques (detailed later in this chapter). However, by analysing the lipid portion after removal of the sugar moiety results in the identification of the position of the unsaturated double bonds providing further information which is not usually reported. The process involves hydrolytic cleavage of the link between the carbohydrate and lipid portions by acid hydrolysis and subsequent derivatization of the resulting fatty acid chains to fatty acid methyl esters analysed by GC or GC-MS [41]. As with most of the techniques in this chapter, a range of different methodologies can be utilised to obtain the same result, in this case fatty acid methyl esters using a number of different derivatisation steps, with only the most commonly reported method [42] detailed below.

Rhamnolipids

1. Take 4 mg of purified glycolipid and dry completely in a sealable sample vial using nitrogen to ensure no traces of water or organic solvents are present.
2. Add 1 ml chloroform, 0.85 ml of methanol and 0.15 ml of sulphuric acid, seal with lid 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. Using a glass Pasteur pipette, remove bottom layer (chloroform layer) containing the fatty acid methyl esters and analyse 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) (*see* **Note 8**).
6. Analytical conditions that can be used are injector temperature 250°C, start oven programme at 50°C, hold for 1 min then ramp at 40°C/min to 215°C and hold for 25 min [43].
7. Electron impact at 70 eV with scan range 50–650 Da and an injection volume of 1 μl.
8. Fatty acid methyl esters retention times increase with chain length and degree of separation (*see* **Note 9**). Modern GC-MS are equipped with databases that can be utilised for identification of each individual fatty acid present.

Sophorolipids

Sophorolipids represent one of the glycolipid groups that contain different isomers that can be accounted for by the position of the unsaturated double bonds in the lipid chain. Since sophorolipids are usually produced with vegetable oil sources, direct analysis of the original oil used during production can provide information on the likely positions of unsaturated double bonds in the lipid portion of the glycolipid. Similar to rhamnolipids a range of different methods have been reported; however, methanolysis is the simplest and can be additionally coupled with derivatisation for improved sensitivity [33], while aldononitrile acetate derivative methods are also reported [44]. Briefly, methanolysis reaction is used to isolate the hydroxy fatty acid portion of sophorolipids [45] and convert them to hydroxyl acid methyl esters, which is the method reported here.

1. Take approximately 30–50 mg of sophorolipid extract, which has been dried using nitrogen, and add 2 ml methanol containing 0.1 M HCl.
2. Heat at 100°C for 40 min.
3. Extract reaction product in separating funnel twice with 5 ml of hexane in 5 ml of presence of 50 g/l NaCl.
4. Dry hexane layer (top layer) under a stream of nitrogen and redissolve in 1 ml of hexane and transfer to GC injection vial.
5. GC-MS conditions as follows, column RTX-5 (Restek) or Carbowax type (Supelco) (*see* **Note 10**). 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 to 320°C, and hold for 5 min.
6. Set MS scan range at 10–350 with the ion source temperature at 200°C.

Trehalose Lipids

GC-MS analysis should be carried out according to procedure in section "Rhamnolipids" [46].

Mannosylerythritol Lipids

GC-MS analysis should be carried out according to the procedure in section "Rhamnolipids" [47].

3.5.2 HPLC Analysis

HPLC is a relatively common analytical technique that can be used to separate glycolipids. Coupling of HPLC with an 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 [46]; however, glycolipids lack chromophoric groups required for UV detection in their native state. To overcome this obstacle, the glycolipids under investigation can be derivatised to *p*-bromophenacyl esters, which can be detected at 244 nm [48, 49]. HPLC-UV and HPLC-ELSD both require a comparison with retention times of standards to allow identification of the structure; however, the presence of isomers cannot be detected. The requirement of standards combined with the inability to observe structural isomers and difficulties that can surround efficient compound derivatisation means that this method is rarely reported in recent times. HPLC-MS is by far the method of choice for chromatographic separation and detection of glycolipid biosurfactants.

HPLC-UV of Rhamnolipids

1. Take a 10 mg of extracted rhamnolipid and dry with nitrogen.
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-bromoaceto-phenone-Et_3N).
3. Heat for 1 hour 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 × 5μm 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 by a change to 50% A over 3 min and held for 10 min (*see* **Note 11**).
7. Set flow rate at 1.0 ml/min with an injection volume of 50 μl.
8. Standard curves of previously purified glycolipids that have similarly been derivatised should be obtained and used to detect and quantify samples based on peak area.

HPLC-ELSD of Rhamnolipids

ELSD allows the detection of glycolipids without the need for derivatisation. ELS detection mechanism is based on the measurement of photons (light) that are scattered by particles of compounds that have been evaporated from the mobile phase.

1. Set up gradient HPLC-ELSD with Inertsil ODS-3 column (Phenomenex, UK) [15].
2. Prepare rhamnolipid sample by dissolving 5mg in 1ml of methanol, filter and place in HPLC vial.
3. Prepare mobile phase A composed of 90:10 (water-acetonitrile) with 0.1% trifluoroacetic acid and mobile phase B consisting of acetonitrile with 0.1% trifluoroacetic acid.
4. Condition column with initial starting condition of 70:30 mobile phase A:B for 30 min.
5. Set up ELSD with a drift temperature of 100°C and a nebulizer flow rate of 1.5 l/min.
6. Analyse samples using the following gradient elution, starting at 30% B going to 70% B by 5 min and then to 90% B by 15 min followed by a ramp to 100% B to clean the column (*see* **Note 11**).
7. Set flow rate at 1.0 ml/min and injection volume 50 µl.
8. Standard curve of pure glycolipid should be used for quantification.

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 [33]; however with ELSD detection, a good separation of the majority of compounds particularly the lactonic structures is achieved using the method detailed by Van Bogaert et al. [50] or the method described below [24].

1. Set up gradient HPLC-ELSD with a C_{18} column, 250 mm × 4.6 mm × 5µm i.d.
2. Prepare sophorolipid sample by dissolving 5 mg in 1 ml of methanol, filter and place in HPLC vial.
3. Prepare mobile phase A of water containing 0.05% formic acid and mobile phase B consisting of acetonitrile.
4. Condition column with initial starting condition of 70:30 mobile phase A:B for 30 min.
5. Set up ELSD with a drift temperature of 60°C and a nebulizer flow rate of 1.5 Bar.
6. Analyse samples using the following gradient elution: starting at 30% B going to 100% B by 56 min (*see* **Note 10**).
7. Set flow rate at 0.8 ml/min and injection volume 40 µl.
8. Standard curve of pure sophorolipid should be used for comparison of retention times and quantification when required.

HPLC-ELSD of Trehalose Lipids

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

HPLC-ELSD of Mannosylerythritol Lipids

MELs are commonly analysed using HPLC-ELSD with normal phase silica columns. Since the majority of MEL production is carried out using soya bean 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 [51, 52]. Using this approach, the relative composition of each of the groups MEL A–D can be obtained without separation of individual congeners. Due to the complexity of MEL's separation using reverse phase HPLC-ELSD would result in an extremely complex chromatogram with many unresolved peaks.

1. Set up gradient HPLC-ELSD with a silica gel column (Nucleosil 100A, 5 μm, 4.6 × 250 mm, Phenomenex, UK) [51].
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 and injection volume 500 μl.
4. Standard curve of pure glycolipid should be used for quantification.

3.5.3 Mass Spectrometry Analysis

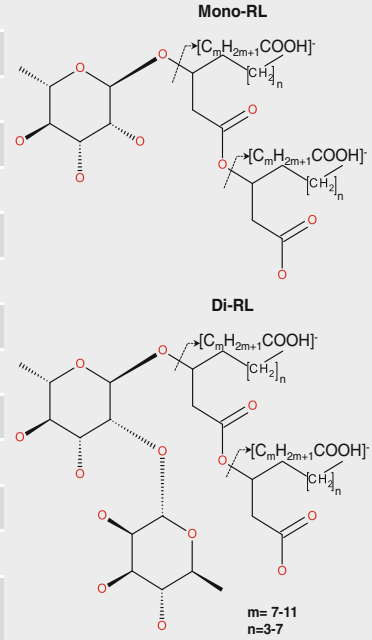
Mass spectrometry (MS) represents a powerful method for the analysis of glycolipids, providing detailed structural information in the molecular mass of the compounds under investigation. Tandem MS (MS/MS) results in the fragmentation of structures thus allowing the identification of individual isomers without the need for separation. Tables 2, 3 and 4 detail ms/ms diagnostic fragmentation information required to identify the different glycolipid structures from each group. In combination with HPLC or UPLC, it provides the most sensitive method for identification and quantification of glycolipids. A drawback, however, is that it requires a reasonably high level of purification as salts and free nonpolar lipids present in the samples can induce suppression of ion signals under MS conditions. Glycolipids can be analysed on all types of mass spectrometers, with electrospray ionisation (ESI-MS) quadrupole-based mass spectrometers and matrix-assisted laser desorption ionisation (MALDI) described here. As with all MS equipment, the experimental technique will vary greatly depending on the manufacturer of the instrument and software; therefore, only specific details on sample preparation and basic operation procedures are described below. Refer to software manuals for specific details on step-by-step operation and software details for operation of equipment.

ESI-MS Quadrupole Ion Trap Analysis of Glycolipids

Electrospray ionisation provides excellent glycolipid ionisation when used for direct infusion or HPLC-MS. Using this technique, virtually no fragmentation occurs in the primary molecules under investigation especially when the cone voltage (sometimes referred

Table 2
Deprotonated molecular ion data for the most commonly found mono- and di-rhamnolipids (typically produced by *Pseudomonas aeruginosa*) along with diagnostic ms/ms fragmentations that can be used for structural identification of each of the main structures and isomeric congeners

Molecular structure	$[M-H]^-$	$[M-H-C_mH_{2m+1}COOH]^-$	Position of major ms/ms fragments on structure
Rha-C_8-C_{10}	475	305	
Rha-C_{10}-C_8	475	333	
Rha-C_{10}-C_{10}	503	333	
Rha-C_{10}-$C_{12:1}$	529	333	
Rha-$C_{12:1}$-C_{10}	529	359	
Rha-C_{10}-C_{12}	531	333	
Rha-C_{12}-C_{10}	531	361	
Rha-Rha-C_8-C_{10}	621	451	
Rha-Rha-C_{10}-C_8	621	479	
Rha-Rha-C_{10}-C_{10}	649	479	
Rha-Rha-C_{10}-$C_{12:1}$	675	479	
Rha-Rha-$C_{12:1}$-C_{10}	675	505	
Rha-Rha-C_{10}-C_{12}	677	479	
Rha-Rha-C_{12}-C_{10}	677	507	

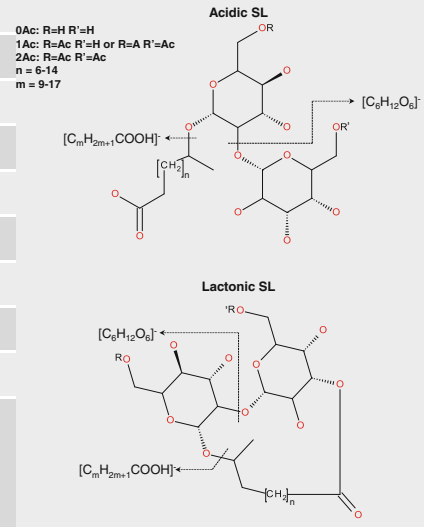


to as sampling voltage) is kept low. Ionised molecules are detected by a mass analyser according to their mass to charge ratio (*m/z*) and can be fragmented using collision-induced dissociation (CID) to provide valuable information about each structure and their isomers. Using an LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, California, USA), the following method is currently used in our laboratory for relatively quick purity and glycolipid composition analysis.

1. Set tune method according to the following conditions: syringe 5 µl/ml, 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 30.0 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.

Table 3
Deprotonated molecular ion data for the most commonly found lactonic and acidic sophorolipids (typically produced by *Starmerella bombicola* previously known as *Candida bombicola*) along with diagnostic ms/ms fragmentations that can be used for structural identification of each of the main structures and isomeric congeners

Molecular structure	$[M\text{-}H]^-$	$[M\text{-}H - C_mH_{2m+1}COOH]$	Position of major ms/ms fragments on structure
Acidic, $C_{18:1}$	621	339	
Acidic, $C_{18:1}$, 1Ac	663	381	
Acidic, $C_{18:2}$, 2Ac	703	423	
Acidic, $C_{18:1}$, 2Ac	705	423	
Acidic, $C_{18:0}$, 2Ac	707	423	
Lactonic, $C_{18:1}$, 1Ac	645	363	
Lactonic, $C_{18:2}$, 2Ac	685	405	
Lactonic, $C_{16:0}$, 2Ac	661	405	
Lactonic, $C_{18:1}$, 2Ac	687	405	
Lactonic, $C_{18:0}$, 2Ac	689	405	



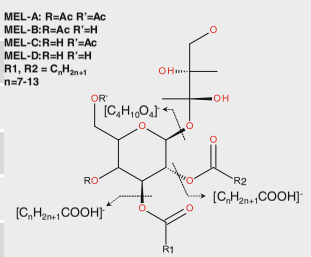
4. Load the sample solution into the syringe, with the fused silica sample tube attached to the syringe. Place syringe in pump and press start.
5. Start scan.
6. Mass spectrum of the sample should appear after a few seconds, change to the appropriate file name and press start to acquire data (*see* **Note 12**).
7. After 1 min, press stop button to finish acquiring the data.
8. Stop scan.
9. Analyse data using the data analysis software.

3.5.4 Liquid Chromatography-Mass Spectrometry Analysis

LC-MS analysis provides in-depth glycolipid analysis combining the polarity-based separation achieved with liquid chromatography with mass separation using advanced mass spectrometry instrumentation. This is by far the most common method of glycolipid analysis and one of the most commonly reported techniques providing detailed compositional and structural analysis of glycolipid. A wide range of different LC- and MS-based instrumentation is currently used for this type of analysis. HPLC is still the most

Table 4

Deprotonated molecular ion data for the most commonly found MELs (typically produced by *Pseudozyma aphidis*) along with diagnostic ms/ms fragmentations that can be used for structural identification of each of the main structures and isomeric congeners

			[M-H-1R]					
	$[M-H]^-$	R1-R2	R1	R2	Loss of R1 + R2	Loss of R1 + $C_4H_{10}O_4-$	Loss of R2 + $C_4H_{10}O_4-$	Position of major ms/ms fragments on structure
MEL A	648.7	$C_{8:0}$-$C_{10:0}$	503.49	475.44	331.23	381.49	353.44	
	724.6	$C_{12:2}$-$C_{12:2}$	527.28	527.28	330.96	405.28	405.28	
	700.6	$C_{10:1}$-$C_{12:1}$	529.34	501.28	331.02	407.34	379.28	
	676.7	$C_{10:0}$-$C_{10:0}$	503.44	503.44	331.18	381.44	381.44	
	728.7	$C_{12:1}$-$C_{12:1}$	529.38	529.38	331.06	407.38	407.38	
MEL B/C	604.6	$C_{8:1}$-$C_{10:0}$	461.39	431.34	289.13	339.39	309.34	
	658.5	$C_{8:0}$-$C_{14:2}$	513.29	433.13	288.92	391.29	311.13	
	658.5	$C_{10:2}$-$C_{12:0}$	489.24	457.18	288.92	367.24	335.18	
	660.5	$C_{10:0}$-$C_{12:1}$	489.24	459.18	288.92	367.24	337.18	
	714.6	$C_{12:0}$-$C_{14:2}$	513.28	489.23	288.91	391.28	367.23	
MEL D	588.7	$C_{10:1}$-$C_{10:1}$	417.44	417.44	247.18	295.44	295.44	
	588.7	$C_{10:0}$-$C10:2$	415.44	419.44	247.18	293.44	297.44	
	616.6	$C_{8:0}$-$C_{14:2}$	471.39	391.23	247.02	349.39	269.23	
	616.6	$C_{10:0}$-$C_{12:2}$	443.34	419.28	247.02	321.34	297.28	
	620.5	$C_{10:0}$-$C_{12:0}$	447.24	419.18	246.92	325.24	297.18	

common LC method; however, with the advanced separating power and speed which can now be achieved with UPLC, a gradual move towards this technique is occurring. Similarly, with mass spectrometry, quadrupole ion trap (QIT), quadrupole time-of-flight (Q-TOF) and matrix absorption laser desorption ionisation time-of-flight (MALDI-TOF) instruments from a range of different vendors are employed for glycolipid analysis. For the purpose of this chapter, LC-MS methods will be highlighted for standard HPLC column, narrow-bore columns and UPLC columns. These LC methods reported can be employed with virtually all mass spectrometry instruments. Similarly, only quadrupole ion trap, Q-TOF and triple quadrupole mass spectrometry methods, along with MALDI, are reported due their frequent use for glycolipid analysis.

HPLC-Quadrupole Ion Trap 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 analysed 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 [16, 22] with the major fragmentation always occurring with the loss of the outer lipid chain. Therefore, the mass of this chain helps to identify the outer chain length and by deduction of the theoretical mass of the rhamnolipid moiety permits the identification of the second lipid chain. A whole host of different mass spectrometers have been reported for the LC-MS analysis of rhamnolipids including simple single quadrupole instruments [53]. For the purpose of the section, only the method for ESI-quadrupole ion trap mass spectrometer is shown below, which is commonly reported for rhamnolipid analysis [12, 17, 54]. The HPLC column and elution parameters could be utilised for any HPLC-MS-based analysis when using the standard HPLC 4.6 mm bore diameter columns.

1. Connect HPLC to LCQ quadrupole ion trap mass spectrometry with ESI source.
2. Adjust tune method nitrogen sheath gas to 65 to evaporate the higher volume of solvent entering the mass spectrometer. Set the auxiliary gas at 35, spray voltage to 4.5 kV, capillary temperature at 250°C, capillary voltage to 30.0 V and tune lens offset to 40 V.
3. Dissolve samples at concentration of 0.1 mg/ml in methanol.
4. Use HPLC with Luna C_{18} column (250 mm × 4.6 mm × 5 μm, Phenomenex) connected.
5. Prepare mobile phase A (water) and B (acetonitrile) and start gradient elution with 70% A in 30% B which was then raised to 70 % mobile phase B after 50 min.
6. Set flow rate at 0.5 ml/min and injection volume of 20 μl/min.

7. 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.

HPLC-Quadrupole Ion Trap 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 [5, 33, 55, 56].

1. Analysis carried out according to section "HPLC-Quadrupole Ion Trap MS of Rhamnolipids" with the following variations.
2. At step 3, use a Gemini (Phenomenex) C_{18} column (250 mm $\times$ 4.6 mm $\times$ 5μm) which produces better separation than the Luna column.
3. At step 5, the gradient conditions should be 50% acetonitrile and 50% water changing to 70% acetonitrile over 50 min.
4. When analysing results, it is necessary to check for other closely related structures, which may be obscured by large peaks. For example, where a major peak has been assigned a structure, search for the same structure with 1 less or one extra double bond in lipid chain, i.e. 2 daltons higher or lower. This is due to the large peak masking minor components of the extract, especially for acidic sophorolipids.

HPLC-ESI-Q-TOF Mass Spectrometry Analysis of Rhamnolipids

Mass spectrometric analysis using a Q-TOF instrument provides information on glycolipid structures through accurate mass measurement of their molecular mass to four decimal places. The data obtained using this instrument, particular in tandem MS mode, permits the identification of the majority of glycolipids. The method detailed below uses a narrow-bore HPLC column resulting in greater resolution, speeds of analysis and could be applied to other MS instruments. The method was developed using a Waters Alliance HPLC coupled to a Waters Q-TOF Premier mass spectrometer

1. Connect an Agilent Poroshell EC-C_{18}, 2.1 $\times$ 100 mm and 2.7 μm particle size column (or equivalent from another supplier) to the HPLC, and set the column oven to 40°C.
2. Set the following parameters on the mass spectrometer tune method: negative mode ionisation, source temperature 120°C, desolvation temperature 300°C, desolvation gas 800 l/h and cone gas 50 l/h. The capillary voltage should be set to 2.6 kV and cone voltage to 30 V.
3. Set up mass spectrometry method performing collision-induced dissociation (CID) of the analytes in MS^e mode with argon by ramping up the collision energy from 20 to 30 eV. Set the mass range of m/z 150–1,200.

4. Dissolve samples at concentration of 0.1 mg/ml in methanol.
5. Prepare mobile phase A of HPLC water (if extra sensitivity is required, add 4 mM ammonium acetate, sonicate to dissolve and filter) (*see* **Note 13**). Mobile phase B consists of HPLC acetonitrile.
6. Set up inlet method with gradient elution using the following method at a flow rate of 0.2 ml/min: 0–10 min, 50–55% B, 10–14 55–70% B; 14–16 min, 70% B, 16–18 min, 70%–50% B; 18–20 min, 50% B.
7. Use an injection volume of 2 μl.
8. Set up sample list using the mass lynx Waters software and run analysis.

HPLC-ESI-Q-TOF Mass Spectrometry Analysis of Sophorolipids

Accurate mass measurement and chemical characterisation of sophorolipids can be carried out using the same methods as per section "HPLC-ESI-Q-TOF Mass Spectrometry Analysis of Rhamnolipids" [57].

UPLC-Triple Quadrupole Mass Spectrometry Analysis of Rhamnolipids

Ultra-performance liquid chromatography has been developed to enable significantly higher system back-pressures than convention HPLC, allowing the use of columns with sub 2 μm particle sizes, resulting in improved chromatographic speed, resolution and sensitivity. Triple quadrupole mass spectrometers are the most sensitive MS instruments available and the most commonly used for quantitative analysis. Combined with UPLC, it provides superior LC resolution and particularly sensitive for glycolipid analysis with incredibly high sample throughputs [18]. When multiple reaction monitoring is employed, detection of individual isomers, which are common in glycolipid extracts, can be identified and quantified as separate peaks. The majority of methods have run times under ten minutes and overcome problems with the relative compositions of individual glycolipid congeners with the same molecular mass. The protocol below is for a rapid (3 min) method developed in-house for a Waters ACQUITY® UPLC system connected to a triple quadrupole (TQD) mass spectrometer (Waters Corporation, Milford, MA).

1. Connect an Agilent Zorbax Eclipse Plus C_{18} UPLC column (2.1 × 100 m, 1.8 μm particle size) to the UPLC system.
2. Set the column temperature to 40°C using the Acquity console manager.
3. Prepare mobile phase A with HPLC water containing 4 mM ammonium acetate, sonicate and filter. Mobile phase B is acetonitrile which should be sonicated beforehand.
4. Set the following parameters on the mass spectrometer tune page: negative mode ionisation, cone voltage to 2.6 kV, source temperature to 120°C, desolvation temperature 350°C, desolvation gas 800 l/h and cone gas to 50 l/h.

5. Set up tandem mass spectrometry in negative ESI mode using multiple reaction monitoring (MRM) method. This should be developed beforehand using the MRM optimisation software IntelliStart (Waters Crop.) (*see* **Note 14**). Optimise for two different transitions ions (usually the loss of the other lipid chains, see Table 2, showing rhamnolipids congeners typically produced by *Pseudomonas aeruginosa*) which will allow the identification of isomeric congeners (*see* **Note 15**).
6. Once the mass spectrometer tune page temperatures have been reached, prime the UPLC according to manual guidelines.
7. Dissolve samples at a concentration of 0.1 mg/ml in methanol.
8. Set up inlet method with the gradient elution using the following method at a flow rate of 0.5 ml/min 0–0.2 min, 50% B; 0.2–2.0 min, 70% B; 2.0–2.2 min, 70%; 2.2–2.3 min, 70–50% B; 2.3–3. 0 min, 50% B.
9. Use an injection volume of 5 μl.
10. Set up sample list using the mass lynx Waters software and run analysis.

UPLC-Triple Quadrupole Mass Spectrometry Analysis of Sophorolipids

UPLC-MS analysis of sophorolipids can be carried out using the same protocol outline for rhamnolipids in section "UPLC-Triple Quadrupole Mass Spectrometry Analysis of Rhamnolipids", using Table 3 for MRM information, with the following modification for the mobile phase gradient.

1. At step 8, run gradient as follows: 0–1.0 min, 50% B; 1.0–8.0 min, 70% B; 8.0–8.5 min, 70%; 8.5–9.0 min, 70–50% B; 9.0–10.0 min, 50% B.

HPLC-Ion Trap MS of MELs

Only one method has been reported, to date, for the LC-MS analysis of MELs using a Bruker ion trap mass spectrometer [58].

1. Connect HPLC to ion trap mass spectrometry with and ESI source.
2. MS condition are as capillary voltage 2.5 kV, capillary temperature at 325°C, cone voltage 40.0 V, desolvation gas 10 ml/min and nebulizer pressure of 40 PSI.
3. Dissolve samples at concentration of 0.1 mg/ml in methanol.
4. Use HPLC with Dionex Acclaim Surfactant column (4.6 mm × 250 mm, 5 μm).
5. Prepare mobile phase A (water containing 0.01 M ammonium formate) and B (acetonitrile), and start gradient elution with 25% A in 75% B and which was then raised to 85% mobile phase B over 25 minutes and then to 100% B in a further 5 min.
6. Set flow rate at 0.4 ml/min and injection volume of 5 μl/min.

3.5.5 MALDI Analysis for All Types of Glycolipids

Matrix-assisted laser desorption ionisation (MALDI) is a soft ionisation mass spectrometry technique connected to a time-of-flight (TOF) analyser that allows the identification of intact compounds. Basically, samples to be analysed 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 analyser (TOF). This characterisation technique has been applied for the analysis of a range of glycolipids including rhamnolipids [41], sophorolipids [44] and trehalose lipids [26].

1. Prepare the matrix, α-cyano-4-hydroxy cinnamic acid (αCHCA), which should be used for glycolipids. Prepare a 10 mg/ml solution of α-CHCA 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 (*see* **Note 16**).

3.5.6 NMR Analysis

The previously described methodologies allow the identification of molecular structure to a reasonable degree of certainty and in general can provide the necessary information when commonly found glycolipids are present. For usual glycolipids or for complete unambiguous identification, NMR can be used. NMR 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.

The glycolipids should be dissolved in deuterated 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 [41, 43, 59], sophorolipids [7, 33–35, 44], trehalose lipids [19, 25, 26] and mannosylerythritol lipids [27, 39, 52] can be obtained from the literature.

4 Notes

1. Use a glycolipid standard appropriate to the biosurfactant under investigation.
2. Under acidic conditions (pH 3.0), the rhamnolipids are present in their protonated form (pK_a 5.6) [60] and are therefore less soluble in water [48].

3. When larger volumes need to be extracted, autoclaving to sterilise without removal of cells may be an advantage.
4. 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.
5. When larger quantities of sophorolipid are produced, sedimentation occurs which complicates the total removal of cells during the centrifugation step.
6. MTBE has also been shown to be a suitable solvent for extraction of trehalose lipids [61].
7. Depending on how much separation is required, increments can range from 2% increases to 10% increases in methanol concentration.
8. Essentially a wide range of columns can be used, and it is best to consult information provided by manufacturers. Due to the limited complexity of the fatty acids present, a specific FAME GC column (usually up to 100 M length) should not be necessary.
9. 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.
10. Other columns and consequently different GC-MS conditions can be used.
11. Other gradient conditions or slight modifications should be used to achieve appropriate separation depending on the column selected.
12. Method shown refers to the LCQ with ESI-MS carrying Excalibur software; for different equipment or software, consult the manufacturers' manuals.
13. Rhamnolipid analysis gradient elution and detection can be carried out using HPLC water and HPLC acetonitrile without mobile phase additives. However, if greater sensitivity is required, the addition of 4 mM ammonium acetate can improve the signal.
14. The majority of triple quadrupole MS instruments have automated MRM optimisation. Consult operation manuals beforehand, and ensure that the correct ms/ms product ions are selected for optimisation (i.e. the loss of the lipid chains only).
15. Prior to developing an optimised MRM method on a triple quadrupole instrument, MS analysis should be carried out in full scan mode to determine the molecular weights of each of the different rhamnolipids present. Individual MRMs should be developed for each rhamnolipid to allow full analysis of the whole rhamnolipid extract during triple quadrupole analysis.
16. Signal is severely influenced by the presence of salts and other impurities.

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Isolation of Glycoprotein Bioemulsifiers Produced by Marine Bacteria

Tony Gutierrez and Ibrahim M. Banat

Abstract

High-molecular-weight (HMW) surface-active agents (biosurfactants and bioemulsifiers) are produced by many different types of bacteria and comprise proteins, glycoproteins, lipoproteins, polysaccharides, lipopolysaccharides or complexes containing any combination of these structural types. The attraction of HMW glycoprotein bioemulsifiers as ingredients in food and drink formulations, for example, has increased in recent years, especially those produced by marine bacteria, as these amphiphilic macromolecules can offer improved emulsifying and emulsion stability properties. Marine bacteria are a largely untapped source for these types of molecules, and unlike those produced by terrestrial bacteria and microalgae, marine bacterial glycoproteins are often highly negatively charged (polyanionic) which endows these macromolecules with potential multifunctional properties. The polyanionic nature and molecular-weight heterogeneity of these types of molecules require careful attention to optimising their isolation from complex media. This chapter provides a detailed description for optimising the isolation/separation of amphiphilic polyanionic glycoprotein bioemulsifiers produced by marine bacteria.

Keywords: Amphiphilic exopolymers, Bioemulsifiers, Biosurfactants, Emulsifiers, Glycoproteins, Isolation, Marine bacteria, Separation, Surfactants

1 Introduction

Emulsifiers are amphiphilic chemical compounds that are used to mix two immiscible substances, such as oil and water. They are used in many industries worldwide, most notably those of food and drink [1], cosmetic and healthcare [2]. Although a significant fraction of the market demand for emulsifiers is met by organochemical synthesis using petrochemicals as precursors, an important trend in the food and healthcare industries is the adoption of 'natural' ingredients with perceived benefits for the consumers' health. Bioemulsifiers, which are produced by biological systems (plants, animals or microorganisms), are generally high-molecular-weight (HMW) polymers that are an attractive substitute for their chemically synthesised counterparts due to their associated

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lower toxicities, higher biodegradability and perceived consumer-friendliness as a natural ingredient. Most, however, are sourced from plants and animals and are often required in large quantities in order to achieve optimal functionality. Microbial-produced bioemulsifiers, in particular those synthesised by bacteria, are commercially promising alternatives to those derived from plants and animals as they are sustainable and have been shown to offer improved properties such as foaming and stabilisation, as well as better tolerance to wider extremes of temperature, pH and salinity [3].

With many years having been devoted to prospecting the biotechnological potential of terrestrial organisms, the marine environment has in only recent times emerged as a highly promising frontier for the discovery of novel natural products, including new types of bioemulsifiers. In essence, our oceans offer a rich and relatively untapped source for these compounds, particularly from bacteria whose collective mass and phylogenetic and genotypic diversity in the marine biosphere far exceed that anywhere else on Earth. Ecologically, bacterial bioemulsifiers serve important functions in marine environments where they may be involved in microbial adhesion to solid surfaces and biofilm formation [4], the emulsification of hydrocarbon oils to enhance biodegradation [5] or mediating the fate and mobility of heavy metals and trace metal nutrients [6, 7]. This wide spectrum of functional activity is reflected not merely in the complex chemistry of these molecules, but also in the diversity of bacterial genera found producing them [8]. A notable feature of these types of bacterial macromolecules is their high uronic acid content, which has been shown to exceed that found in exopolymers produced by marine microalgae and non-marine (aquatic and terrestrial) bacteria [9]. The high content of these acids in marine bacterial exopolymers renders them highly polyanionic (negatively charged) and thereby quite reactive [10]. Some polyanionic macromolecules exhibit amphipathic properties that allow them to interact with oily substrates [11], in some cases enhancing the dissolution of the oils by the process of emulsification [5, 11]. Their amphipathic nature is often attributed to the presence of a hydrophobic component(s) attached to the polysaccharide backbone, such as the fatty acids of RAG-1 emulsan [12], or protein of some glycoprotein emulsifiers [13, 14].

Most bacterial bioemulsifiers are glycoproteins [3, 15] and recent work has shown that marine bacteria are a viable source of glycoprotein bioemulsifiers. These protein-polysaccharide conjugates are highly attractive as ingredients in, for example, food and drink formulations because they offer improved emulsifying and emulsion stability properties [16] compared to their artificially constructed counterparts. The uronic acid moieties of these macromolecules can confer them with an ability to interface with

hydrophobic organic chemicals, such as food oils and petrochemical hydrocarbons [6, 11]. Similarly, the amino acid and/or peptide groups of these exopolymers have also been shown to confer amphiphilic characteristics to these macromolecules [11, 17]. In addition, bioemulsifiers in general have antibacterial activity yet mostly low toxicity [18]. Bacterial-derived HMW glycoproteins contain O-glycosidic links that act as the predominant bonding mechanism of the glycan to the protein [19], via acidic amino acids or -OH groups. The enhanced functionality of bacterial HMW glycoproteins is attributed to the hydrophobic regions of the protein constituents, and possibly also uronic acids, serving here as anchoring points to help attach the large bulky hydrophilic carbohydrate regions to emulsion oil droplet surfaces. This forms a very thick, adsorbed layer around the emulsion droplet that is extremely efficient as an emulsion stabiliser, preventing droplet coalescence and emulsion breakdown. Since marine bacterial HMW glycoproteins are often highly polyanionic, careful attention is needed for optimising their isolation in order to increase their yield.

In this chapter, a method is described for isolating extracellular HMW glycoprotein bioemulsifiers from marine bacteria. Whilst the method can be used and/or adapted for isolating these compounds from almost any bacterial strain, it has been optimised for isolating these types of highly polyanionic exopolymers.

2 Materials

All chemicals can be purchased from any laboratory chemical supplier. My lab generally uses Fisher Scientific (http://www.thermofisher.com) and Sigma-Aldrich (http://www.sigmaaldrich.com).

2.1 Preparation of ZM/1 Media

1. Natural filtered seawater: Collected from a coastal or offshore site, ideally far from industrial inputs of contamination. Filter the seawater using Whatman 1 filter paper and store at 4°C (*see* **Note 1**).
2. Trace elements stock solution: Dissolve 4.36 g Na_2EDTA, 3.15 g $FeCl_3 \cdot 6H_2O$, 0.022 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.18 g $MnCl_2 \cdot 4H_2O$ and 6.3 mg $Na_2MoO_4 \cdot 2H_2O$ in 100 ml of deionised water and filter sterilise (0.2 μm). Store at 4°C.
3. Vitamin stock solution: Dissolve 0.5 mg cyanocobalamin (B_{12}), 100 mg thiamine HCl (B_1) and 0.5 mg biotin in 100 ml of deionised water and filter sterilise. Store in the dark at 4°C.
4. Marine supplements stock solution: Combine 1 ml of the trace elements stock solution, 5 ml of the vitamin stock solution and

94 ml of deionised water; autoclave (121°C, 15 min) or filter sterilise. Store at 4°C.

5. Bacto-peptone: purchased from most suppliers of microbiological media, such as Thermo Scientific Oxoid.
6. Yeast extract: purchased from most suppliers of microbiological media, such as Thermo Scientific Oxoid.

2.2 Preparation of Glucose Stock Solution

1. Glucose: can be purchased in small quantities (e.g. 500 g) or in bulk (several kilograms) and cheaply from most laboratory chemical suppliers (*see* Subheading 3.2).

2.3 Growth of Marine Strains in Liquid Culture for Production of Glycoprotein Bioemulsifiers

1. A temperature-controlled rotary shaker incubator: Any conventional model can be used as long as it can accommodate glass Erlenmeyer flasks (*see* Subheading 3.3).
2. Erlenmeyer glass flasks (2–5 L capacity): purchased from most suppliers of laboratory equipment (*see* **Note 2**; *see* Subheading 3.3).

2.4 Monitoring Production of Bioemulsifying Glycoproteins by Marine Strains

1. Glass screw-cap test tubes (100 × 13 mm) (*see* Subheading 3.3.2).
2. A vortex device: Most common laboratory types can be used with a speed range of 200–3,200 rpm (*see* Subheading 3.3.2).
3. For the emulsification assay: a hydrocarbon (e.g. *n*-hexadecane) or food-grade oil (e.g. sunflower) is used as the oil substrate (*see* **Note 8**; *see* Subheading 3.3.2).

2.5 Isolation of Glycoprotein Bioemulsifiers

1. For bulk removal of cell biomass: a common bench-top or floor standing centrifuge with a speed of up to 8,000 rpm to pellet bacterial cells.
2. For complete removal of bacterial cells: a filtration apparatus that can accommodate 0.2 μm filters. For large volumes, a cross-flow filtration system would be ideal.
3. Distilled water: ideally of 18 MΩ/cm quality to minimise the introduction of trace metal ions that could interact with charged groups of the polymer.
4. For dialysis of the unrecovered polymer: a cross-flow filtration system that can accommodate a membrane cassette of a desired molecular-weight cut-off (e.g. 5, 10, 30, 50 or 100 kDa).
5. For precipitation of the polymer: potassium chloride (KCl) and cold 99% ethanol.
6. For dialysis of the recovered polymer: dialysis tubing of a desired molecular-weight cut-off (e.g. 5, 10, 30, 50 or 100 kDa).
7. For freeze-drying (lyophilisation) of the polymer: a freeze dryer.

3 Methods

The protocol described below provides a description for isolating extracellular glycoprotein bioemulsifiers from marine bacteria. Various parameters can significantly influence the recovery and quality of exopolymer extracts during downstream processing. With respect to charged exopolymers, such as HMW glycoproteins produced by marine bacteria, the ionic strength of the cell-free permeate has been shown to influence the yield of these macromolecules that is recovered [11]. The HPLC chromatograms shown in Fig. 1 illustrate the influence of ionic strength on the heterogeneity of a marine bacterial biopolymer fraction in spent medium. Under low ionic strength conditions (i.e. 18 MΩ/cm quality water), these macromolecules exhibit the tendency to complex, hence forming high-molecular-weight aggregates that are eluted as three poorly resolved peaks within the void volume (>2,000 kDa) of the HPLC

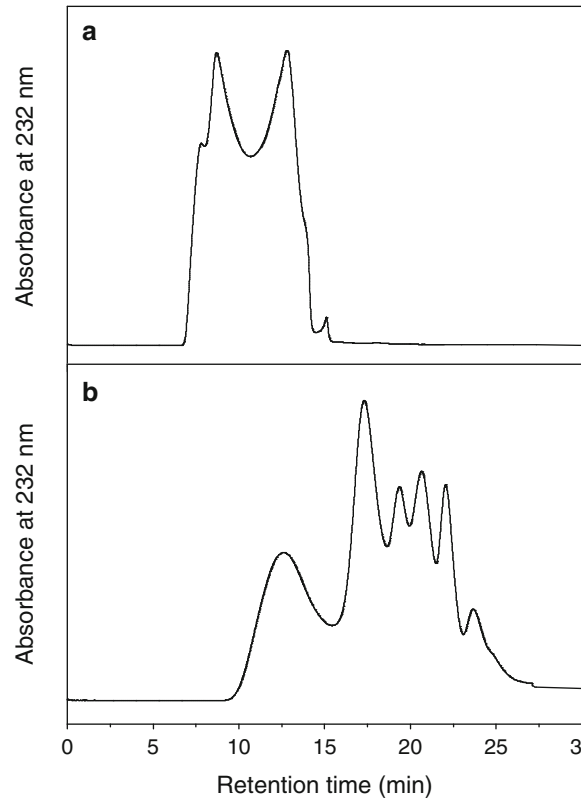

Fig. 1 HPSEC analysis of a polyanionic glycoprotein biopolymer produced by a marine bacterial strain eluted through an Ultrahydrogel 2000 size-exclusion column with 18 MΩ/cm quality water (**a**) or filtered synthetic seawater (**b**) as eluent [11]. These chromatograms show how ionic strength can alter the polydispersity (i.e. molecular-weight heterogeneity) of these biopolymers

column (Fig. 1a). However, under high ionic strength conditions, such as that reminiscent in bacteriological media used to grow marine bacteria, these aggregates exist in dissociated form as lower-molecular-weight macromolecules. As shown in Fig. 1b, these dissociated species resolved into six distinct peaks. Excluding the first two peaks (>2,000 and 247 kDa, respectively), the remaining four peaks represent macromolecules of molecular weights less than 100 kDa which would become lost in the filtrate volume during the ultrafiltration step (*see* Subheading 3.4).

It is important to note that the liquid chromatography analysis should be performed using a mobile phase that closely resembles the ionic strength of the growth medium, as it would reveal a more accurate measure of the purity and composition, including molecular weight (M_r) of these macromolecules (*see* **Note 3**). It would also shed greater insight into ways that could optimise their recovery and increase yields. Hence, during the recovery of these macromolecules from bacterial spent medium, reducing the ionic strength of the cell-free filtrate (after removal of the biomass) is a key initial step in the recovery of these macromolecules prior to their subsequent precipitation with an appropriate solvent, such as ethanol or *iso*-propanol. Although most protocols proceed directly to solvent extraction immediately following removal of the cells from the spent medium, this could effectively lead to partial or even major losses in polymer yields. Since exopolymers produced by marine bacteria are recognised as highly polyanionic [9, 10] and which can exhibit molecular-weight heterogeneity (i.e. polydispersity index values > 1) (*see* **Note 4**), these polymers will exist in seawater ionic strength conditions (e.g. 0.6 M NaCl) as non-aggregated molecules spanning a wide molecular-weight range. This salt-induced heterogeneity is attributed to the high content of uronic acids in these charged macromolecules because these acids tend to ionise at seawater pH and salinity and cause the low-molecular-weight species to dissociate and exist freely in solution [20]. Their precipitation out of solution during the solvent-extraction step can lead to the precipitation of also unwanted material, such as media components and various other non-target materials, which could persist as contaminants in subsequent steps of recovery and purification of the exopolymers. Here we describe a method for isolating polyanionic glycoprotein bioemulsifiers to optimise their recovery from seawater-based (high ionic strength) media by incorporating a dilution step prior to their recovery by ultrafiltration and solvent-mediated precipitation. By reducing the ionic strength of the cell-free permeate prior to ultrafiltration, the recovery of these charged macromolecules is maximised.

3.1 Preparation of ZM/1 Medium

ZM/1 medium is based on Zobell's 2216 marine medium, as previously described [21]. Whilst this medium has been shown to be optimal for producing glycoprotein bioemulsifiers from some

strains of marine bacteria (*see* Subheading 3.3), different concentrations of peptone and yeast extract can be tested to determine which ratio of these ingredients might lead to higher glycoprotein yields and/or that yield higher bioemulsifier activity (*see* **Note 5**). As described below (*see* Subheading 3.3), we use 750 mL of ZM/1 broth in each 2 L glass culture flask. To prepare 1 L of this medium, dissolve 5 g peptone and 1 g yeast extract in 250 ml water, and then add 750 ml filtered natural seawater. Dispense the required volume into each culture flask (750 mL per 2 L flask) and autoclave (121°C, 15 min). Store at 4°C. When you are ready to use this medium, aseptically add 3.75 ml of the marine supplements and 15 ml of glucose stock (1% w/v final concentration).

3.2 Preparation of Carbon Sources

1. Glucose stock solution (50% w/v): Dissolve 50 g in 100 ml water and autoclave (121°C, 15 min). Store at room temperature (*see* **Note 6**; *see* Subheading 3.3).

3.3 Growth of Marine Strains in Liquid Culture for Production of Glycoprotein Bioemulsifiers

Depending on the strain type, we have found ZM/1 medium amended with glucose (to 1% w/v final concentration), or a modified version of this medium (e.g. ZM/10), to produce optimal yields of glycoprotein bioemulsifiers from marine bacteria [6, 22–24]. However, the choice of sugar substrate should be guided from preliminary experiments testing a variety of sugars (incl. other feedstock substrates) for their ability to serve as a suitable carbon source that will yield the highest quantities of the target glycoprotein bioemulsifier(s). Different media types, including varying the concentrations of media components (e.g. peptone, yeast extract, etc.), should also be evaluated for this purpose. The method described below for isolating glycoprotein bioemulsifiers from marine bacteria utilises 2 L glass Erlenmeyer flasks containing 750 ml of ZM/1 medium. However, most types of glass culture vessel can be used (*see* **Note 7**). If using glass flasks with screw caps, be sure to keep the caps loosely fitted during incubation in order to allow for the diffusion of air and keep the cultures well aerated.

3.3.1 Growth of the Strains in Liquid Medium for Bioemulsifier Production

1. Inoculate the flask(s) with 5–10 mL of a growing culture of the bacterial strain to be used.
2. Incubate the flasks on a shaker (150–250 rpm) at a selected temperature. We generally use an incubation temperature of 25–28°C, but preliminary tests using a range of incubation temperatures will guide the selection of the optimal temperature that will yield high bioemulsifier production rates and yields.
3. From the point of inoculation (designated time zero), it will be necessary to monitor for bioemulsifier production activity on a daily basis. For this, an appropriate emulsification assay will be required (*see* Subheading 3.3.2).

4. Once maximal emulsification values have been reached (*see* Subheading 3.3.2), commence to isolate the glycoprotein bioemulsifying product from the spent medium (*see* Subheading 3.4).

3.3.2 Assaying for Bioemulsifier Production During Growth

During growth of the bacterial strain in batch culture flasks, or in a fermenter vessel under optimised and controlled conditions, it is important to monitor for the production of the bioemulsifier in order to determine when to terminate growth (i.e. when maximal bioemulsifier yields are reached) and then commence isolating the bioemulsifier from the spent medium. Various analytical methods, such as liquid chromatography [6, 11, 23], can be used to quantify bioemulsifier production, but often such methods require expensive equipment and may not necessarily be high throughput. A simple, cost-effective and rapid method to assay for bioemulsifier production would be advantageous, and for this we use two types of assays. Each is differentiated for the emulsion type formed – i.e. a water-in-oil (W/O) or oil-in-water (O/W) emulsion – as conferred by the structural and physicochemical characteristics of the bioemulsifier. The oil substrate used in the emulsification assay can also influence the emulsion type formed; we generally use *n*-hexadecane as a standard oil substrate for these assays (*see* **Note 8**). Here we describe two simple methods to assay for the production of bioemulsifiers that produce W/O and/or O/W emulsions, respectively, during growth of a bacterial strain in liquid medium.

Assaying for Bioemulsifiers That Produce W/O Emulsions

This assay has been adapted from the method of Cooper and Goldenberg [25].

1. Pipette 2 mL of the cell-free (i.e. cells removed by centrifugation and/or filtration; *see* Subheading 3.4) spent medium into each of three glass screw-cap tubes (100 × 13 mm).
2. Then pipette an equal volume (2 mL) of *n*-hexadecane into each of the three replicate tubes – another oil substrate can be used if appropriate.
3. Screw the caps tightly on the tubes, and before proceeding to mix both layers in the next step, use a ruler to measure the height of the oil layer. Also take a look at the interfacial boundary between the oil and water interface to see if it appears curved upward or has become straightened – if the latter, this is an indication that the spent medium contains an amphiphilic (emulsifying or surface-tension reducing) agent.
4. Vigorously mix both layers in each of the three tubes. Though there are any number of ways to do this, we use a quick and simple method that involves initially shaking the tube manually by hand for approximately 15 s, then vortexing for another 15 s to homogeneity and finally repeating this mixing step once more.

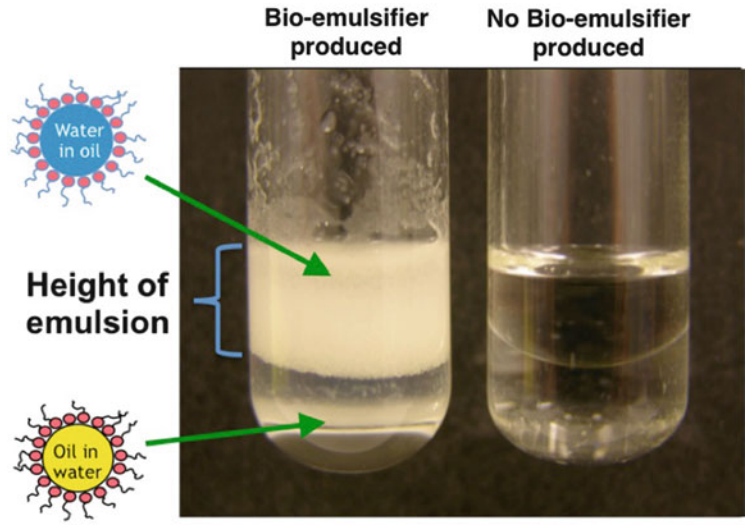

Fig. 2 The emulsification assay performed by mixing an oil substrate, such as *n*-hexadecane, with an equal volume of cell-free spent medium (*lower phase*) obtained from a culture of a bacterial strain that synthesises extracellular glycoprotein bioemulsifier (*left tube*). The bioemulsifier presented here produces a water-in-oil (W/O) emulsion (*upper layer*) and a O/W emulsion (*lower layer*). The Emulsification index (EI) is expressed as a percentage of the height of the emulsion layer to the total original height of oil in the tube. The result of a control assay is included (*right tube*) to show no emulsification of the oil

5. Allow the emulsions to stand for 10 min before repeating the previous shaking step once more.
6. Allow the emulsions to stand for a final 10 min and then measure the height of the emulsion layer in each tube (Fig. 2; *see* **Note 8**). The height of the emulsion is then divided by the initial height of the oil (before emulsification) to give a value for the Emulsion Index (EI). The value can be expressed as a percentage when multiplied by 100. The assay is semi-quantitative – higher values are indicative that the spent medium sample contains higher concentrations of bioemulsifier.

Assaying for Bioemulsifiers That Produce O/W Emulsions

This assay has been adapted from the method of Cirigliano and Carman [26].

1. Conduct the emulsification assay, in triplicate, as described in **steps 1–5** of Subheading 3.3.2 above, with the only modification that 2.5 ml of spent medium is mixed with 0.4 ml of *n*-hexadecane.
2. After allowing the emulsions to stand for 10 min, spectrophotometrically measure the turbidity at 540 nm of the bottom

aqueous layer in each tube and every 10 min thereafter for up to 60 min.

3. The log of the turbidity values is then plotted against time. The slope of the curve is expressed as the decay constant, K_d, and is a measure of the stability of the O/W emulsions formed.
4. A final turbidity reading is measured from each tube after allowing the emulsions to stand for 24 h, and the values expressed as the emulsifying activity (A_{540}).
5. Higher K_d and A_{540} values are indicative that the spent medium sample contains higher concentrations of bioemulsifier.

3.4 Isolation of Glycoprotein Bioemulsifiers from the Spent Medium

The following method was developed for the optimised isolation of glycoprotein bioemulsifiers from spent media, as described in Gutierrez et al. [11]. The method is based on the fact that these types of marine bacterial polymers are highly polyanionic and heterogeneous mixtures of varying molecular weight. Reducing the ionic strength of the spent medium encourages these macromolecules to aggregate, hence increasing their molecular weight to, for example, >100 kDa. In this way, a high-molecular-weight cut-off dialysis membrane (e.g. of 100 kDa pore size) can be utilised for their recovery. This is advantageous for the removal of lower-molecular-weight contaminants derived from the growth medium or produced by the bacterial strain whilst also maximising the recovery of the target high-molecular-weight bioemulsifying fraction. The method below employs the use of a cross-flow ultracentrifugation system for this initial recovery step as it is a simple and easy apparatus to use (*see* **Note 9**).

1. Remove the cell biomass from the spent medium, the latter of which will contain the desired bioemulsifying fraction. There are a number of techniques that can be used to do this, for which one of the most efficient is cross-flow filtration using a 0.2 μm membrane cassette. If this apparatus is unavailable, one can collect the bulk of the cell biomass by centrifuge (8,000–10,000 × *g*; 20 min) and then use filtration (0.2 μm) to remove the remaining bacterial cells from the spent medium.
2. Dilute the cell-free permeate (containing the bioemulsifying fraction) by at least threefold using distilled 18 MΩ/cm quality water – this is to reduce the ionic strength and promote the polyanionic bioemulsifying macromolecules to aggregate. Aggregation will not lead to precipitation of these macromolecules out of solution.
3. The diluted cell-free permeate is then passed through a 100 kDa membrane cassette by cross-flow ultrafiltration, and during this process, the retentate volume is dialysed with at least 5 L of distilled (preferably 18 MΩ/cm quality) water.

4. The retentate volume, which contains the bioemulsifying fraction in partially purified form, is then precipitated using KCl (7.5% w/v) and 3–4 volumes of cold 99% ethanol (*see* **Note 10**). Respectively add the KCl first and then the ethanol, and do so gradually in order to minimise creating high concentration zones of both chemicals in the retentate solution.
5. Allow the precipitate to settle overnight at 4°C before recovering it by centrifugation at low speed (3,000–5,000 rpm; 10 min).
6. The wet precipitated material will need to be dialysed against 18 MΩ/cm quality water in order to remove KCl, other salts and low-molecular contaminants that accompanied the bioemulsifying polymer. For this, we generally use snake-skin dialysis tubing of a 1 kDa molecular-weight cut-off. The precipitated material is placed inside the dialysis tubing (make sure to follow the manufacturer's instructions on how to prepare the membrane for use) and dialysed against 10–20 L of the deionised water. Depending on the size of the container available to do this, you may need to replace the water with a fresh volume on a daily basis.
7. Depending on how you want to use this now partially purified biopolymer, you can store it either at 4°C for immediate use or with a preservative (e.g. sodium azide to 0.005–0.01% w/v final concentration) for use over a few weeks. We generally freeze-dry the polymer so that it can be stored for long term. A conventional freeze dryer obtained from most suppliers selling laboratory equipment will suffice for this purpose. The wet (in solution) or lyophilised polymer can be analysed by HPSEC in order to assess the molecular-weight heterogeneity of its macromolecular constituents.

4 Notes

1. Natural seawater may not be easily accessible to some research groups that live far inland from a coast. In such circumstances, natural seawater can be purchased from some marine microbial repositories and aquariums, or a synthetic powdered version can be sourced from most suppliers of bacteriological media.
2. The type of culture flask should accommodate either autoclavable screw caps or cotton wool or foam plugs to allow for aeration during incubation.
3. For this we use high-performance size-exclusion chromatography (HPSEC). Most types of liquid chromatography that are equipped with a refractometer and UV detector will suffice. We use a Waters Ultrahydrogel 2000 column (7.8 × 300 mm;

Waters Ltd) at 30°C. The eluent should simulate the ionic strength of the growth medium and the resultant chromatograms compared to those generated using other ionic strength conditions. A flow rate of up to 1 ml/min can be used. To calibrate the column for molecular-weight estimation, we generally use dextran standards of M_r range 12,000–1,800,000 Da.

4. Polydispersity index (I_p) is a measure of the distribution of different molecular weights within a polymer sample and is the ratio of the weight average molecular weight (M_w) and the number average molecular weight (M_n). For a comprehensive description and how to measure this value, see Harding et al. [27].
5. Other versions of ZM/1 medium could be used to test whether they influence the rate of production and yields of bioemulsifiers from the bacterial strains. We have found that for some strains, lower concentrations of the peptone and yeast extract in ZM/1 can lead to higher bioemulsifier yields, but more often higher concentrations of these ingredients lead to higher growth rates, biomass and bioemulsifier yields.
6. Under autoclave conditions, other sugars and sugar-based substrates may be susceptible to degradation, caramelisation or the effects of the Maillard reaction. In such cases, sterilisation by filtration (0.2 μm) is recommended.
7. A fermenter system can be used, but it is often recommended to do so after culture flask experiments have been performed to determine the culture conditions for optimisation of bioemulsifier production.
8. Food-grade oils, such as those derived from plant and animal sources, inherently contain emulsifying agents that will contribute emulsifying activity. It is therefore important, particularly when using a food-grade oil substrate in the emulsification assay, to always include a negative control in order to take into account any emulsification contributed by the oil. To avert this, we often use *n*-hexadecane (>99% purity) as the oil substrate in our emulsification assays. Nonetheless, we still include a negative control, which constitutes mixing of the oil with the uninoculated medium.
9. It is appreciated that the purchase of a cross-flow (ultra)filtration system can be quite expensive and therefore not affordable to some research groups. An alternatively cheaper approach would be to use YM-100 Centricon filter units (Millipore, Dundee, UK), although these will handle substantially smaller volumes compared to the cross-flow. Depending on the scale of the cross-flow system available, it can handle volumes from several litres to 10s of 100s of litres.

10. Ethanol is a chaotropic agent that interacts with charged moieties of exopolysaccharides and disrupting the hydrogen bonding of water molecules, which leads to the precipitation of these biopolymers from solution. *Iso*-propanol is a replacement solvent for ethanol when the biopolymers are to be used in applications related to food and drink.

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Protocols on Lipid Extraction from Wet Algal Biomass

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Abstract

Lipids from microalgae present a promising feedstock for biodiesel production, but deployment for economical production requires cost reductions for microalgae cultivation, dewatering, and lipid extraction. Lipid recovery from wet concentrated algal slurry, rather than from dried microalgae, is therefore desirable to avoid the need for secondary dewatering and/or drying. This chapter provides two protocols to enable extraction of cellular lipids from wet algal biomass. Depending on the cell wall composition, pretreatment of biomass is recommended prior to lipid extraction to facilitate better solvent penetration. While the chloroform/methanol-based Bligh and Dyer method may give higher lipid recovery yields, the use of safer solvents and in particular solvent combinations, such as a mixture of hexane and ethanol, may be more suitable for large-scale extraction.

Keywords: Biodiesel, Biofuel, Lipid extraction, Microalgae, Microalgal slurry, TAG, Triacylglycerol

1 Introduction

Microalgae have been considered a promising feedstock of biodiesel based on their high areal productivity and their ability to grow in most types of water (fresh, brackish, seawater, and even wastewater) while not requiring arable land [1–3]. However, their cultivation, harvesting, and downstream processing require cost reductions to enable economical production of microalgal fuels and by-products [4]. One of the main contributors of costly downstream processes of microalgal biodiesel production is dewatering and drying of the biomass [5–7]. In many of the current lipid extraction/recovery techniques, moisture can hinder the process efficiency significantly. The main reason is that moisture avoids efficient contact between microalgal cells and recovery solvent [8, 9]. On the other hand, most microalgal cells are composed of rigid cell walls which prohibit the access of recovery solvent to the intracellular lipid contents. To overcome all these obstacles, a process should be developed in which microalgal cells are ruptured in their wet condition and their lipids recovered by a suitable solvent mixture (Fig. 1).

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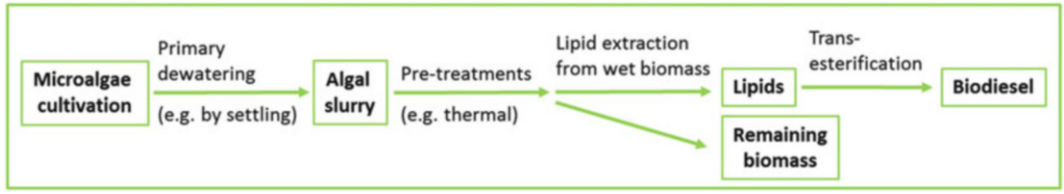

Fig. 1 Biodiesel from microalgae: integration of lipid extraction from wet biomass to avoid costs for secondary dewatering and drying

First, to avoid dealing with large volumes of water, primary dewatering or concentrating of microalgae is required (e.g., by settling [10]). Among others, thermal and microwave-assisted pretreatments have been shown to be effective in rupturing or weakening microalgal cell walls [8, 11]. In some microalgal species with weak cell walls, osmotic shock can be applied to open up the cells [12]. After applying pretreatments, the lipid bodies of the microalgal cells can be recovered through applying a suitable solvent. Target compound polarity is the main factor determining the suitable solvent. We and others have previously shown that mixing polar and nonpolar solvents can enhance the lipid recovery efficiency [9, 11]. Increasing the polarity of the solvent not only facilitates the solvent penetration into the microalgal cells but also results in better access to microalgal lipids when moisture is present. In this protocol, we will describe and compare two solvent recovery techniques: (1) Bligh and Dyer's technique [13] that is widely used in laboratories but not considered practical for large-scale use as it involves chloroform and (2) a solvent mixture of hexane and ethanol (3:1 ratio).

2 Materials

- Hexane, methanol, chloroform, and ethanol; all were HPLC grade sourced from Merck KGaA
- 40 mL glass vials with polytetrafluoroethylene (PTFE)-lined caps
- Corex glass centrifuge tubes
- 2 mL soda glass tubes
- Avanti centrifuge (HP-20 XPI) (Beckman Coulter) for centrifugation of volumes higher than 1 L
- Techno Spin R centrifuge (Sorvall Instruments) for less than 100 mL volumes
- Vacuum desiccator
- Digital Mettler AM50 scale with 0.1 mg accuracy

- Agilent 6890 GC coupled to a 5975 MSD
- LG Microwave Oven Model no. MS3447GR
- Hot plate/magnetic stirrer

3 Methods

3.1 Lipid Extraction and Gravimetric Measurement

Microalgal slurry (e.g., from *Scenedesmus* sp.) is prepared after primary dewatering of microalgal culture that has been stimulated for cellular triacylglycerol accumulation in lipid bodies to result in a 16 g dry weight/L mixture. Alternatively, microalgal slurry can be reconstituted for experimental purposes by mixing biomass harvested by centrifugation with water (paste/water ratio of 1:5). After applying suitable pretreatments (e.g., microwave-assisted or thermal; see notes), 8 mL of the algal slurry is placed in a 40 mL glass vial with PTFE (polytetrafluoroethylene)-lined caps.

3.1.1 Extraction via Bligh and Dyer Method

For wet lipid extraction according to an adapted Bligh and Dyer method, 5 mL chloroform and 10 mL methanol are added to 8 mL algal slurry, and the mixture is vortexed for 4 min. Then additional 5 ml chloroform is added, followed by vortexing for another 4 min (ratio of chloroform/methanol/water, 2:2:1.8). Then the content of the vial is transferred to Corex glass centrifuge tubes followed by centrifugation at 1,000 × *g* for 7 min to form phase separation. At least five times, 2 mL of the organic layer (chloroform with extracted lipids on bottom) is pipetted and transferred to five pre-weighted soda glass tubes, and the chloroform is evaporated using a vacuum desiccator.

3.1.2 Extraction via a Solvent Mixture

Volumes of 15 mL hexane and 5 mL ethanol (3:1 ratio) are added to 8 mL algal slurry, followed by 4 min of vortexing. Then the vial content is transferred to Corex glass centrifuge tubes and centrifuged at 1,000 × *g* for 7 min for phase separation. At least five times, 2 mL of the organic layer (hexane with extracted lipids on top) is pipetted and transferred to five pre-weighted soda glass tubes, and the hexane is evaporated using a vacuum desiccator.

3.1.3 Calculating Total Amount of Extracted Lipids

Weighing the samples and gravimetric measuring of the lipid extractions are carried out using a digital Mettler AM50 scale with 0.1 mg accuracy, subtracting the weight of each soda glass tube.

3.2 Fatty Acid Analysis

Around 2 mg of extracted lipids are redissolved 1:1000 in chloroform, and a 100 μL aliquot is taken and dried down. Then, the lipids are hydrolyzed and methyl esterified with 300 μL of 2% H_2SO_4 in methanol solution at 80°C by shaking (480 rpm) for 2 h on a thermal mixer. Prior to esterification, 50 μg of heneicosanoic

acid (C21) is added to the pellet in each sample as an internal standard. After esterification, 300 μL of 0.9% (w/v) NaCl and 300 μL of HPLC grade hexane are added and vortexed for 20 s. Phase separation is performed by centrifugation at 16,000 × *g* for 3 min, and the hexane layer is used for fatty acid methyl ester profile analysis by gas chromatography/mass spectroscopy (GC/MS). GC/MS analyses are carried out on an Agilent 6890 GC coupled to a 5975 MSD using 1 μL injection. A DB-Wax column (Agilent, 122-7032) is used with running conditions as described in Agilent's RTL DB-Wax method (Application note: 5988-5871EN). Identification of fatty acid methyl esters is based on mass spectral profiles and retention times in the Agilent's RTL DB-Wax method.

4 Notes

4.1 Pretreatments

4.1.1 Thermal Pretreatment

Microalgal slurry is transferred to an Erlenmeyer flask and subsequently placed on a hot plate with a magnetic stirrer. The culture has to be heated up to 80°C temperature and kept at this temperature for 10 min while it is continuously stirred.

4.1.2 Microwave-Assisted Pretreatment

A beaker is filled with microalgal slurry and placed in a microwave (LG Microwave Oven Model no. MS3447GR) at a setting of 1.1 kW for 3 min. The slurry is then stirred to a homogenous mixture and immediately placed in the microwave under the same settings for another 2 min. The temperature of the slurry after the microwave treatment ranges typically between 80°C and 90°C.

4.2 Precautions To Be Taken During Transferring of the Organic Layer

In lipid recovery by hexane and ethanol, the centrifugation results in the hexane layer on top, microalgal debris in the middle, and a mixture of water and ethanol in the bottom of the Corex tube. In this case, pipetting the hexane layer is straightforward. However, for the Bligh and Dyer method, the phase separation after centrifugation is different with a mixture of water and methanol on top, algal debris in the middle, and the chloroform organic phase in the bottom layer of the Corex tube. In this case, extra care should be taken when pipetting the chloroform layer to avoid water and methanol and more importantly algal debris. To make this happen, as soon as the pipet reaches the top layer of water and methanol, positive pressure should be applied to the pipet which causes bubbling in the tube, and this pressure should be released upon the contact of the pipet with the bottom chloroform layer.

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Isolation and Characterization of Lipid Droplets from Yeast

Karin Athenstaedt

Abstract

Lipid droplets are cell compartments serving as a depot for non-polar lipids, mainly triacylglycerols and steryl esters. Furthermore, these compartments are involved in lipid metabolism, signal transduction, membrane biosynthesis and trafficking, as well as storage of proteins, and additional functions may be discovered in the future. A crucial prerequisite for detailed studies of lipid droplets is the availability of a highly purified cell fraction. Here, a protocol for the isolation of lipid droplets from the model organism yeast *Saccharomyces cerevisiae* is presented. Characteristics of a lipid droplet fraction are a limited number of proteins and a high content of non-polar lipids. Most common problems occurring during isolation of lipid droplets and advices to overcome these problems are included.

Keywords: Lipid droplet, Non-polar lipids, *Saccharomyces cerevisiae*, Steryl esters, Triacylglycerols, Yeast

1 Introduction

Human defects leading to aberrant accumulation of non-polar lipids are associated with several severe diseases of modern civilization such as diabetes type II, obesity, and arteriosclerosis. Thus, from the medical point of view, excessive accumulation of non-polar lipids has to be prevented. In contrast, scientists working in the field of biotechnology seek for targets to increase the cellular non-polar lipid content, because in industry organisms accumulating huge amounts of non-polar lipids are employed for, e.g., single-cell oil production or the production of biofuels. To manipulate the cellular non-polar lipid content in either way requires not only a detailed knowledge about the formation and mobilization of storage molecules but also about the cell compartment where non-polar lipids are deposited – the lipid droplet. Lipid droplets, also known as lipid particles, adiposomes, oleosomes, or oil bodies, are formed in all eukaryotic organisms, e.g., mammals, plants, and fungi, as well as in a few prokaryotes of the gram-positive genera (for reviews see, e.g., [1, 2]). The basic structure of lipid droplets is

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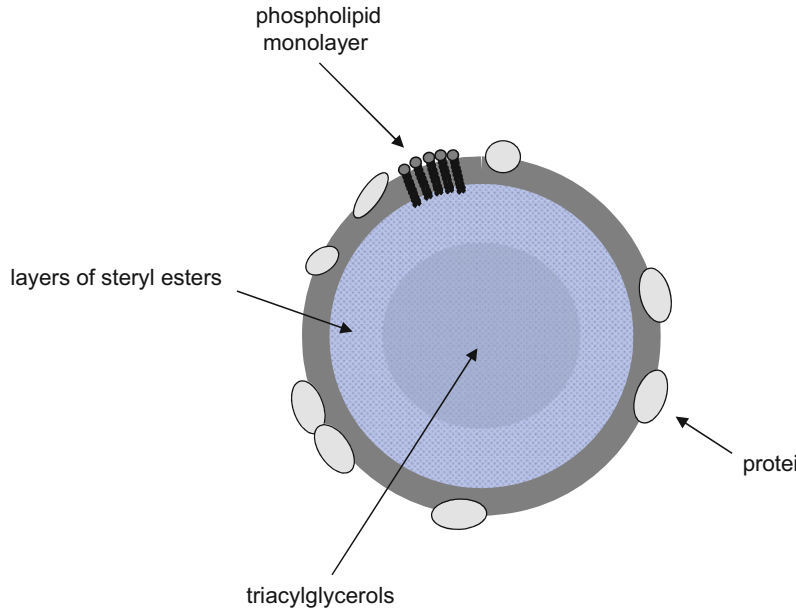

Fig. 1 Scheme of a lipid droplet. A phospholipid monolayer circumvents the hydrophobic core of a lipid droplet, which is formed of triacylglycerols and several layers of steryl esters [3]. Additionally, a few characteristic proteins are embedded into the phospholipid monolayer

rather simple. Non-polar lipids are forming a hydrophobic core, which is encompassed by a phospholipid monolayer. Additionally, a few characteristic proteins are embedded into the monolayer of the droplet (Fig. 1).

The main non-polar lipids stored in lipid droplets are triacylglycerols and steryl esters. The ratio of these two lipids strongly depends on the cell type forming the droplet. As examples, in adipocytes the hydrophobic core of lipid droplets mainly consists of triacylglycerols, whereas steryl esters are the main component of these storage compartments present in steroidogenic cells (reviewed in [1]). A respectable part of our current knowledge about the role of lipid droplets in cell metabolism and their structure comes from studies performed in the model organism yeast *Saccharomyces cerevisiae*. In this yeast the ratio of triacylglycerols and steryl esters stored in lipid droplets is approximately 1 when glucose serves as a carbon source [4]. This ratio drastically increases in the presence of oleic acid, because this carbon source not only enhances triacylglycerol formation but simultaneously decreases synthesis of steryl esters [5, 6]. However, independent of the composition of the hydrophobic core and the source of lipid droplets, more than 95 % of the droplet weight account to lipids and the remaining portion to the proteome of this organelle. Due to the

high lipid content, the density of lipid droplets is rather low compared to other organelles. Cell fractionation procedures developed for the isolation of lipid droplets take advantage of the low density and separate these storage compartments from other organelles by flotation on density gradients. "Separation by flotation" is also the principle of the procedure presented here, which describes the isolation of lipid droplets from the budding yeast *S. cerevisiae*. This protocol was developed more than 20 years ago by Leber et al. [4]. These authors observed that lipid droplets tended to stick to vacuoles in intact cells and during isolation. Therefore, a protocol for the isolation of vacuoles was chosen as a basis, adapted at several points, and finally Leber et al. succeeded in having established a method which yielded a highly purified fraction of lipid droplets.

2 Isolation of Lipid Droplets: Step by Step

The following protocol is optimized for the isolation of lipid droplets from the budding yeast *S. cerevisiae*. If it is intended to isolate lipid droplets from other yeast species, it may be necessary to adapt this protocol at certain steps. Descriptions of adapted procedures for the isolation of lipid droplets from the oleaginous yeast *Yarrowia lipolytica* and from the methylotrophic yeast *Pichia pastoris* can be found in Athenstaedt et al. [7] and Ivashov et al. [8], respectively.

2.1 Cell Growth

In rapidly dividing cells the high demand of membrane lipids for cell proliferation leads to predominant formation of polar lipids. However, at later growth phases non-polar lipids such as triacylglycerols and steryl esters are the main products of lipid synthesis. As mentioned above, these non-polar lipids form the hydrophobic core of lipid droplets and serve as a source of energy and/or building blocks for membrane formation in the absence of nutriments. Therefore, a higher number of lipid droplets can be observed in cells from the late exponential growth phase compared to cells from an early growth phase. The highest number of lipid droplets is present in stationary cells. However, since spheroplasting cells from the late stationary phase is rather difficult, it is recommended to use cells grown to the late exponential/early stationary phase for lipid droplet isolation.

A second parameter influencing the number of lipid droplets per cell is the growth medium. Microscopic inspections revealed that the number of lipid droplets is significantly increased in cells grown on synthetic media compared to cells cultivated on full

medium (YPD, consisting of 2 % glucose, 1 % yeast extract, 2 % peptone [9]).

2.2 Solutions and Specific Equipment

2.2.1 Solutions

(a) For spheroplast preparation:

Buffer A: 0.1 M Tris/SO_4^{2-} (pH 9.4) + 10 mM dithiothreitol (freshly added)

Warning: dithiothreitol is harmful; wear protective gloves!

Solution B: 1.2 M sorbitol–20 mM KH_2PO_4 (pH 7.4)

Zymolyase (e.g., 20,000 U/g offered by amsbio; www.amsbio.com)

(b) For lipid droplet preparation (*see* **Note 1**):

Buffer C: 10 mM 2-morpholinoethanesulfonic acid (MES)–Tris (pH 6.9)–12 % (w/w) Ficoll 400–0.2 mM EDTA

Buffer D: 10 mM MES–Tris (pH 6.9)–8 % (w/w) Ficoll 400–0.2 mM EDTA

Buffer E: 10 mM MES–Tris (pH 6.9)–8 % (w/w) Ficoll 400–0.6 M sorbitol–0.2 mM EDTA

Buffer F: 10 mM MES–Tris (pH 6.9)-0.25 M sorbitol–0.2 mM EDTA

1 M PMSF (phenylmethanesulfonyl fluoride) in DMSO (stock solution)

Warning: PMSF is acutely toxic; wear protective gloves!

All buffers are stable at 4°C for several months (buffer A without the addition of dithiothreitol). However, solution B gets easily contaminated.

A PMSF stock solution can be stored at −20°C for several months. Since only small amounts are needed, 1 ml of the PMSF stock solution is sufficient for several preparations.

2.2.2 Required Specific Equipment

Low speed centrifuge with cooling function

Ultracentrifuge

Swing-out rotor for 100,000 g centrifugation and the respective tubes (*see* **Note 2**)

Different sizes of Dounce homogenizers (e.g., a 40-, 15-, and 5-ml homogenizer) and the respective loose fitting pestles

2.3 From Culture to Isolated Lipid Droplets

Like other cell fractionation procedures, lipid droplet preparation starts with the formation of spheroplasts. In the following, spheroplasts are broken and lipid droplets are separated from other cell components by several gradient centrifugation steps.

Part 1: Preparation of Spheroplasts (According to Daum et al. [10]):

Cells grown to the late exponential/early stationary phase are harvested by centrifugation [3,000 g, 5 min; (*see* **Note 3**)] and once washed with water. To prepare the cells for spheroplasting, the cell pellet is suspended in buffer A (0.1 M Tris/SO_4^{2-}; (pH 9.4) + 10 mM dithiothreitol (freshly added)) to a final concentration of 0.5 g CWW/ml and subsequently shaken for 10 min at 30°C. After washing the cells one time with solution B (1.2 M sorbitol–20 mM KH_2PO_4; (pH 7.4), cells are treated with zymolyase dissolved in solution B (40 U/g CWW; 6 U/ml) at 30°C in a shaking water bath. Generally, spheroplasting of pretreated cells takes between 30 and 60 min depending on the growth phase of the harvested cells and the culture medium used. The obtained spheroplasts are gently washed twice with solution B (cooled on ice) prior to homogenization.

Continue immediately with the steps described in part 2.

Part 2: Preparation of Lipid Droplets (Leber et al. [4]):

All following steps are to be carried out on ice.

The washed spheroplasts are suspended in buffer C (10 mM MES–Tris (pH 6.9)–12 % (w/w) Ficoll 400–0.2 mM EDTA) to a final concentration of 0.5 g CWW/ml. To inhibit proteases PMSF from a stock solution is added to the suspension to a final concentration of 1 mM. Spheroplasts are homogenized with a Dounce homogenizer by applying 20 to 30 strokes using a loose fitting pestle. Subsequently the homogenate is centrifuged at 5,000 g for 5 min at 4°C. The resulting supernatant is transferred into a centrifuge tube appropriate for ultracentrifugation and overlaid with at least an equal volume of buffer C. The overlaid sample is centrifuged for 60 min in a swing-out rotor at 100,000 g (4°C). The floating layer obtained after centrifugation has to be collected from the top of the gradient and to be resuspended very gently in buffer C by using a homogenizer and a loose fitting pestle. The suspension is again transferred into a tube suitable for ultracentrifugation, overlaid with buffer D (10 mM MES–Tris (pH 6.9)–8 % (w/w) Ficoll 400–0.2 mM EDTA) and centrifuged for 30 min at 100,000 g (4°C). The obtained floating layer is removed from the top of the gradient and suspended very gently in buffer E (10 mM MES–Tris (pH 6.9)–8 % (w/w) Ficoll 400–0.6 M sorbitol–0.2 mM EDTA) by using a homogenizer (loose fitting pestle). For the last centrifugation step, the suspended sample already enriched in lipid droplets but still contaminated with vacuoles is overlaid with buffer F (*see* **Note 4**) (10 mM MES–Tris (pH 6.9)–0.25 M sorbitol–0.2 mM EDTA). After a final centrifugation for 30 min at 100,000 g (4°C), a white layer is obtained which contains highly purified lipid droplets.

The whole procedure of lipid droplet isolation including the preparation of spheroplasts takes approximately 6–7 h. When glucose serves as a carbon source, an average yield of ~25 μg lipid droplet protein per gram CWW can be expected.

Table 1
Comparison of lipid droplet isolation protocols

	Lipid droplet isolation according to	
Criterion	**Leber et al. [4]**	**Ding et al. [11]**
Time required	6–7 h	6–7 h
Culture volume	≥1.5 l	400 ml
Yield	~25 μg/g CWW	~25 μg in total
Cell disruption	Spheroplasting and homogenization	French press
Separation by	Density gradient centrifugation	Density gradient centrifugation

Recently an alternative method for the isolation of lipid droplets from yeast was presented by Ding et al. [11]. Similar to the procedure presented here, this method separates lipid droplets from other cell compartments by repeated density gradient centrifugations.

Table 1 provides a short summary and comparison of the two cell fractionation protocols.

3 Quality Control of the Obtained Lipid Droplet Fraction

The quality and efficiency of lipid droplet isolation can be determined by protein analyses (*see* **Note 5**) as well as analyses of the lipid composition of the obtained cell fraction.

The molecular mass of lipid droplet proteins ranges between ~30 and 70 kDa leading to a simple but very characteristic protein pattern (Fig. 2). Major deviations in this protein pattern may indicate protein degradation or significant cross-contamination of the obtained lipid droplet fraction with other organelles. The reason for a changed protein pattern can be assessed by, e.g., Western blot analyses.

The proteome of lipid droplets from *S. cerevisiae* wild-type cells grown under standard conditions consists of approximately 50 polypeptides ([5, 12]; www.yeastgenome.org). However, only a limited number of these proteins are exclusively localized to the droplet and thus fulfill the criterion of a marker protein. Commonly, antibodies directed against Erg6p and Erg7p – two prominent lipid droplet proteins catalyzing steps of the ergosterol biosynthetic pathway – are used in Western blot analyses to demonstrate the identity of the isolated fraction. However, it has to be kept in mind that in cells harvested from an early growth phase, a significant portion of these polypeptides is similarly present in the endoplasmic reticulum. Additionally, Western blot analyses

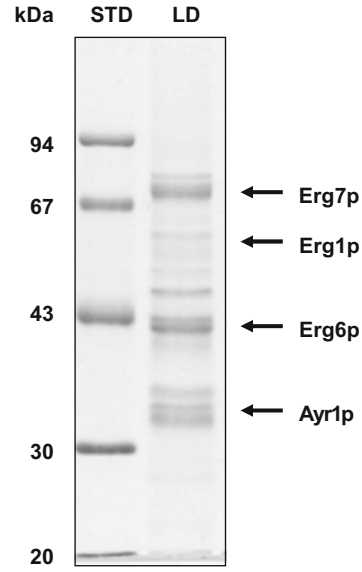

Fig. 2 Characteristic protein pattern of isolated lipid droplets from *Saccharomyces cerevisiae*. The proteome of lipid droplets from *Saccharomyces cerevisiae* consists of a limited number of proteins ranging in size between ~30 and 70 kDa. Some prominent lipid droplet proteins are indicated. *Ayr1p* 1-acyl-dihydroxyacetone phosphate reductase; *Erg1p, Erg6p, and Erg7p* enzymes catalyzing steps of the ergosterol biosynthetic pathway; *LD* lipid droplet fraction; *STD* molecular mass standard

can be used to determine the efficiency of lipid droplet isolation by calculating the enrichment factor of a lipid droplet specific protein over homogenate. By following the protocol of lipid droplet isolation described above, an enrichment factor of 130–140 can be expected [4].

Alternatively, the quality of a lipid droplet fraction can be determined by analyzing the lipid composition. As mentioned above, lipid droplets are characterized by a high non-polar lipid content, whereas the amount of phospholipids is less than 5 % of the droplet weight (Table 2). A significantly augmented amount of phospholipids signals contamination with other organelles.

4 Notes

1. Since dissolving of Ficoll 400 takes a long time, the respective buffers should be prepared in advance.
2. The use of clear ultracentrifugation tubes facilitates the observation during preparation of the gradients.

Table 2
Composition of lipid droplets from *Saccharomyces cerevisiae*

A		B	
Component	**Weight %**	**Phospholipid**	**% of total phospholipid**
Proteins	2.6	PC	36.4
Triacylglycerols	51.2	PI	31.6
Steryl esters	44.4	PE	20.0
Sterols	<0.3	PS	5.4
Squalene	0.5	Dimethyl-PE	3.9
Phospholipids	1.3	PA	2.7

PA phosphatidic acid, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine
Data from Leber et al. [4]

3. To obtain sufficient material for subsequent analyses, it is recommended to use at least 10 g of cell wet weight (CWW) for lipid droplet preparation.
4. Preparation of the third gradient is facilitated by filling buffer F into the tube first and then putting the lipid droplet suspension to the bottom of the tube with the aid of, e.g., a syringe.
5. The high non-polar lipid content of a lipid droplet fraction affects procedures for protein analyses. Therefore, these fractions have to be delipidated prior to, e.g., protein quantification and gel electrophoresis. Lipids from the lipid droplet fraction can be removed by extracting the sample with 3 volumes of diethyl ether. After 20 min of extraction, the organic phase is withdrawn and residual solvent removed under a stream of nitrogen. An alternative method which uses chloroform and methanol to delipitate the lipid-rich fraction is described in Wessel and Flügge [14]. Moreover, when using lipid droplet samples to analyze the protein pattern by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) it is recommended to dissociate these samples at 37°C (30 min) to avoid hydrolysis of polypeptides which may occur at higher temperatures.

Table 3
Troubleshooting

Problem	Possible reason	To check	Remedy
Low yield	Cells were harvested prior to non-polar lipid accumulation	Growth characteristic	Grow cells to the late exponentional/early stationary phase
	Lipid droplet formation is generally poor in the strain used	Check lipid droplet formation by, e.g., fluorescence microscopy (NileRed® staining), analysis of the non-polar lipid content	Use more cells for lipid droplet isolation
	Inefficient spheroplast formation	Check the progress of spheroplasting by microscopy and/or photometrically	Increase the time of zymolyase treatment and/or add more zymolyase per gram CWW
	Inefficient homogenization of spheroplasts		Increase the number of strokes for homogenization and/or repeat the step of homogenization
Impurity	The ratio of suspension to buffer for the gradient centrifugation steps is too high		Reduce the ratio of suspension to buffer for the gradient centrifugation steps and/or repeat the last gradient centrifugation step

5 Troubleshooting (Table 3)

Most common problems occuring during isolation of lipid droplets are a low yield and impurity of the isolated fraction. In Table 3 potential reasons for these problems and advices to overcome these problems are presented.

6 Research Outlook

Lipid droplets formed in *S. cerevisiae* wild-type cells grown in full medium (YPD) are quite uniform in size with a mean diameter of 0.3–0.4 μm [4]. During cell proliferation these droplets form two subgroups in respect to their motility [15]. One subgroup moves from a more central position to the cell periphery but remains within the mother cell. The second group, however, is transferred into the daughter cell during the budding process. The finding that lipid droplets are asymmetrically distributed between mother and daughter cell suggests different features of the lipid droplet subpopulations. One major challenge for the future is the development of an isolation procedure which allows separating these two droplet populations. Analyses of the lipidome and proteome of the isolated subpopulations may reveal the reason for the different destinies of lipid droplets and thus immensely contribute to our current understanding of the role of these organelles in cell metabolism.

Besides inheritance, lipid droplets are formed de novo. Even though the details of lipid droplet biogenesis are still elusive, evidence accumulated that these cell compartments are formed from the endoplasmic reticulum. During this process a specific set of proteins is transferred from the endoplasmic reticulum to the nascent droplet [13]. Interestingly, most of these proteins are involved in lipid metabolism [12]. Another common feature of polypeptides forming the lipid droplet proteome is the presence of hydrophobic stretches whereas transmembrane domains are generally lacking. The finding that hydrophobic stretches are required but not sufficient for the attachment of polypeptides to the droplet [16] suggests the existence of a short targeting signal and/or posttranslational modifications which finally direct a protein to the droplet. As an example, attachment of one or more fatty acids to a polypeptide would increase its hydrophobicity and thus facilitate the anchoring to the lipid droplet surface. Detailed analyses of lipid droplet proteins will be required to determine the selection criteria for the transfer to the droplet.

As mentioned above, among the polypeptides forming the lipid droplet proteome, a high number are involved in lipid metabolism ([5, 12], www.yeastgenome.org). This finding indicates active participation of lipid droplets in lipid turnover. Additionally, these compartments have been shown to be involved in signal transduction, membrane biosynthesis, and vesicle trafficking (for reviews, see [17, 18]). Similarly, a function as a "parking lot" for proteins is discussed for this organelle. Having in mind that several lipid droplet associated proteins await their identification and characterization, it is tempting to speculate that additional cell metabolic roles of these compartments will be discovered in the future.

These few examples already illustrate that quite different aspects can be addressed by studying a highly purified lipid droplet fraction. Thus, a procedure yielding highly purified lipid droplets represents a crucial tool to contribute to a better understanding of lipid droplets and their role in cell metabolism.

Acknowledgement

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Protocols for Isolation and Analysis of Polyhydroxyalkanoates

Mònica Bassas-Galià

Abstract

Some storage compounds such as polyhydroxyalkanates have been drawing considerable attention due to their similar properties to those of conventional plastics. Therefore many efforts have been done to develop and improve analytical methods to isolate and characterize these biopolymers. This chapter pretends to give easy guidelines in all the steps for recovering and characterizing these compounds.

Keywords: Biopolymers, Chromatography, Extraction, Hydrolysis, Microscopy, NMR, Polyhydroxyalkanoates, Purification

1 Introduction

The accumulation of different lipophilic compounds as intracellular inclusions is a phenomenon extensively known in many prokaryotic organisms. The formation of these storage compounds is promoted in response to stress conditions (e.g., nutrient limitation or environmental conditions) [1]. Triacylglycerols (TAGs), wax esters (WEs), or polyhydroxyalkanoates (PHAs) are just few examples of these storage compounds that in the last decades have prompted an increasing biotechnological interest [2]. Polyhydroxyalkanoates are optically active bacterial polyester produced by a large number of bacteria as intracellular granules under metabolic stress conditions. The PHA's composition is dependent on different factors such as the enzymatic specificity or the substrate supplied during the biosynthesis. A large number of carbon sources have been used (alkanes, alkenes, alcohols, fatty acids, waste residues, etc.), and thereby a wide variety of polyesters have been described. PHAs were first reported as a storage compounds in 1926 by Lemoigne [3, 4]. Since then, several methods have been developed for a better understanding of the morphology and composition of these lipidic inclusions. It has been widely described that the monomer

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composition of PHAs is variable and strongly depends on the substrates used and the cultural conditions. As far as the properties of these materials and consequently future industrial applications depend completely on the monomer composition, it has been necessary a technological development of old and new methods to isolate and characterize these polymers. The appearance of powerful spectroscopies (NMR, FTIR, etc.) and other analytical techniques (HPLC, HPLC/MS, GC, GC/MS or GC/MS/MS, GPC, DSC, TGA, etc.) has played an important role in the determination of the monomer composition and in the material properties characterization of PHA. In this chapter, it will be described the very basic protocols for the downstream process and chemical characterization of PHAs, from the intracellular granule to the final biopolymer.

2 Materials

2.1 Microscopical Approaches (see Note 1)

1. Nile red (Sigma, ref. N3013) solution (0.25 mg mL^{-1} in DMSO (Sigma, D8418)) (Sigma-Aldrich, http://www.sigmaaldrich.com).
2. Isotonic solution, $MgCl_2$ (10 mM), filtered or sterilized by autoclaving (it is not necessary but recommended) (Sigma, http://www.sigmaaldrich.com).
3. Glutaraldehyde (4% and 2%) and formaldehyde (5%) solution.
4. Phosphate buffer solution (PB, 0.1 M, pH 7.4): 13.96 g K_2HPO_4, 2.69 g KH_2PO_4 per 1 L, pH 7.4. All reagents can be purchased in Sigma-Aldrich.
5. TE buffer: TRIS (20 mM), EDTA (1 mM), pH 6.9. All reagents can be purchased in Sigma-Aldrich.
6. Cacodylate buffer: cacodylate (0.1 M), $CaCl_2$ (0.01 M), $MgCl_2$ $6H_2O$ (0.01 M), sucrose (0.09 M), pH 6.9.
7. Poly-L-lysine precoated coverslips (Sigma-Aldrich).
8. Osmium tetroxide, potassium hexacyanoferrate(II) trihydrate, uranyl acetate, and lead citrate were purchased in Sigma-Aldrich (http://www.sigmaaldrich.com).
9. Centrifuge with cooling for Eppendorf tubes.
10. Critical point drying with CO_2 system.
11. Sputter coater system.
12. Ultramicrotome.

2.2 PHA Recovery

1. During the DSP (polymer extraction and purification), different solvents (e.g., dichloromethane, methanol, hexane, ethyl acetate, chloroform, etc.), and acid solutions (sulfuric acid and hydrochloric acid) are required and can be easily purchased

(e.g., Acros Organics, http://www.acros.com). Additional basic reagents, sodium sulfate anhydrous, sodium chloride, or sodium carbonate might be needed but all of them can be purchased in Sigma-Aldrich, for example.

2. High-speed centrifuge.
3. Lyophilizator (−80°C, 0.2 mbar).
4. Rotatory evaporator.
5. Polymer extraction: For small-volume samples, the extraction can be performed in glass culture Pyrex tubes with screw cap (www.sigmaaldrich.com, Ref. Z653594). For larger samples, and depending if temperature is required, a Soxhlet system can be used instead. Otherwise, when the extraction is at room temperature, normal Schott-Duran™ lab bottles (agitation is recommended during the extraction) can be used instead [5, 6].
6. Filter paper. In the case of big-volume or high-cell-density samples, pressure filtration system is recommended (e.g., Ref. 16274, Sartorius Stedim Biotech).
7. Hydrolysis and derivatization reactions: All reagents needed for the reactions described in Sect. 3.2.4 can be easily purchased in Sigma-Aldrich (www.sigmaaldrich.com). All the reactions can be carried out in the Pyrex glass tubes with screw cap described above.
8. Polymer derivatization-silylation reaction: Nowadays there exists a large list of silylation reagents. In the Sect. 3.2.5, we recommend the use of hexamethyldisilazane (Fluka, Ref. 52619) and chlorotrimethylsilane (Fluka, Ref. 89595). Both of them can be purchased in Sigma (www.sigmaaldrich.com)

3 Methods

3.1 Microscopical Approaches

3.1.1 Fluorescence Microscopy: A Qualitative Determination of PHA Granules, Staining with Nile Red

Some different dyes such as Sudan black, fuchsin, Nile blue A, or Nile red have been used for the observation of intracellular granules. Nile red presents an orange–red fluorescence with an excitation and emission wavelengths at 542 and 598 nm, respectively. Moreover, the staining with Nile red allows the direct observation of the hydrophobic inclusions with the fluorescence microscope and the adequate filter (e.g., Filter UMF2; 350/488/594). Since it is not a PHA-specific staining, Nile red only determines the existence of lipidic inclusions but is not distinguishing composition. Therefore this dye could be used for different types of lipophilic inclusions (e.g., WEs or TAGs). The lipidic depots appear as intensive red spots, while the rest of the cell is almost not stained (Fig. 1).

It is possible with fluorescence microscopy to follow not only the physiological state of the cells but also the PHA accumulation

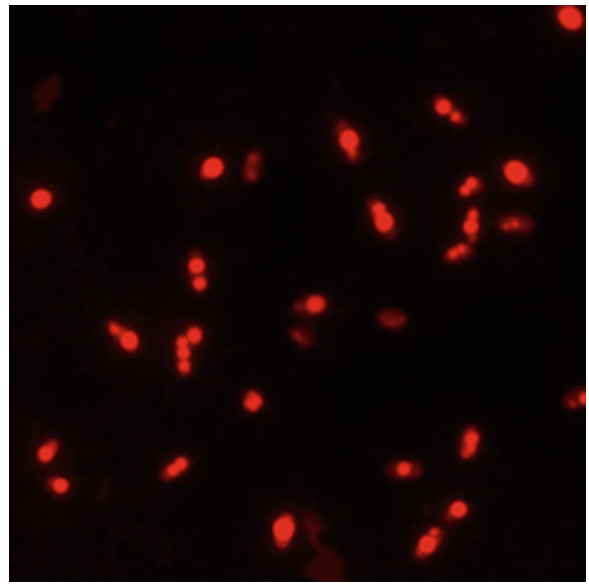

Fig. 1 Nile red staining of *Pseudomonas aeruginosa* 42A2 cells grown on mineral medium (MM1) and undecenoic acid (1% (v/v)) as carbon source. PHA inclusions appear as intensive *red spots*

rate and yield [7–9]. Thus this method is useful, especially, in kinetics studies [10, 11]. Samples are prepared as described below:

1. Disposed 2 mL of culture in an Eppendorf tube (*see* **Note 2**).
2. Addition of two drops of the Nile red solution.
3. After agitation, the sample is centrifuged at 14,000 rpm, at 4°C for 5 min.
4. The supernatant is discarded and the pellet is washed twice with 2 mL $MgCl_2$ (10 mM) to eliminate the excess of Nile red.
5. The pellet is resuspended in $MgCl_2$ (10 mM). Final volume depends on the cell density of each sample
6. 5–10 μL of sample is disposed on a microscope slide and cover with a coverslip. The sample is ready for microscopy.

3.1.2 Scanning Electron Microscopy (SEM)

This microscopy gives information about cell morphology and physiological state of the bacterial cells. It is possible with this technique to observe changes in the cell morphology due to the granule formation (*see* Fig. 2) [12].

Samples are prepared as it is described below (*see* **Note 3**):

1. 1 mL of culture is disposed in an Eppendorf tube and 1 mL of 2% glutaraldehyde and 5% formaldehyde solution in TE buffer is added (1:1 ratio (v/v)).
2. After 2 h at 4°C, the samples are centrifuged at 2,000 rpm, at 4°C for 5 min.

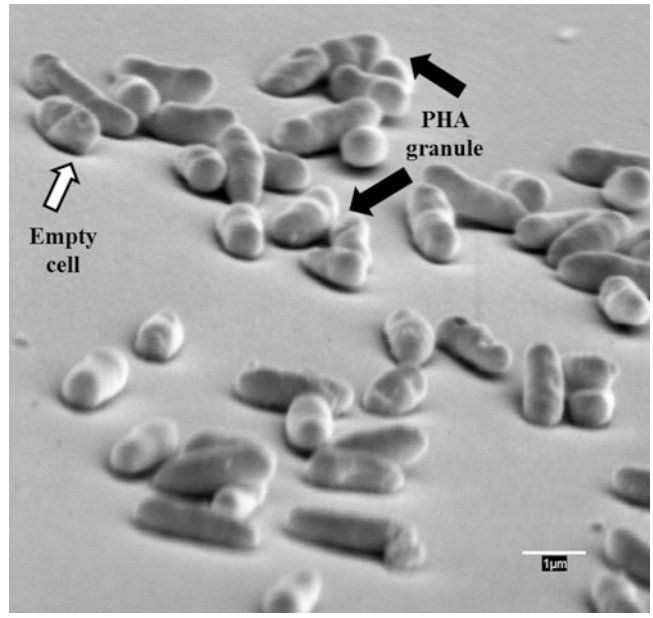

Fig. 2 SEM image of *P. aeruginosa* 42A2 grown in minimum mineral media (MM1) and using Weichol (industrial waste that contains 80% of oleic acid) as a carbon source. The image was acquired in a Zeiss DSM 940A microscope with an accelerating voltage of 10–15 kV

3. After fixation, cells are washed with cacodylate buffer first and then with TE buffer.
4. 50 μL of washed bacteria was applied to poly-L-lysine precoated coverslips (12 mm diameter) and left for 5 min, washed again with TE buffer, and incubated with glutaraldehyde (2%) in TE buffer for 15 min and washed again with the TE buffer. The adhesion time of the sample is around 10 min [13, 14]
5. Dehydration is carried out with a graded series of acetone (10, 30, 30, 50, 70, 90, 100%) on ice for 15 min each step, followed by 100% acetone at room temperature and critical-point drying with liquid CO_2.
6. Samples are then gold shadowed by sputter coating.
7. Samples are ready to be examined.

In Fig. 2, it is shown how the intracellular polymer granule formation in *P. aeruginosa* 42A2, in most of the cases, is deforming the membrane of the bacterial cells.

3.1.3 Transmission Electron Microscopy (TEM)

TEM analysis of ultrathin sections has been widespread used for the characterization of the structure of bacterial cell and its content. The adequate preparation of biological samples for electron microscopy is critical in order to preserve the original structure and to prevent the artifact formation [11, 15–18].

1. Samples are fixed using glutaraldehyde (2%) and formaldehyde (5%) as it has been described in step 2 of the Sect. 2.1.
2. The pellet is washed with cacodylate buffer.
3. Cells were then postfixed with 1% (w/v) osmium tetroxide solution containing 0.8% (w/v) of $K_4Fe(CN)_6$ for 1.5 h at 4°C. The Eppendorf tube is covered with aluminum paper.
4. After 1.5 h, the pellet is washed additionally three times (10 min, 4°C) with 100 mM PB or bidistilled Milli-Q water.
5. Cells are dehydrated through a graded acetone series (50–100%), infiltrated at room temperature in an acetone/Spurr mixture at 3:1 for 1 h, 2:2 for 3 h, and 3:1 overnight.
6. Embedded in fresh Spurr resin (EM Ltd.) for 5 h and polymerized for 48 h at 60°C [19].
7. The ultrathin sections were cut with a Leica UCT ultramicrotome. Ultrathin sections (30–90 nm thickness) were deposited on a copper grid.
8. To obtain a better contrast, an additional staining is recommended. Sections were post-stained with 2% (w/v) aqueous uranyl acetate solution for 30 min. After washing with distillated water, the sample is contrasted again with Reynolds solution (lead citrate) for 10 min [20].
9. The sample is ready to be examined.

In Fig. 3 some examples are shown. The micrographies correspond to *P. aeruginosa 42A2* cells from different culture conditions. The different color of PHA inclusions is due to the substrates used and the olefinic content of the polymer.

3.2 PHA Recovery

To extract, quantify, and characterize the polymer, preliminary steps are needed such as lyophilization (better for polymer extraction), purification, and in some cases a further chemical derivatization to enhance and facilitate the structural analysis.

3.2.1 Lyophilization of the Sample (See Note 4)

1. The culture is centrifuged (8,000 rpm, 4°C, 15 min) and the supernatant is discarded.
2. The cells have to be washed with water to eliminate the remaining salts from the medium. In the cases that the carbon sources are oily substances, often it is necessary to wash the cells with a mixture of H_2O/ hexane (10:1 (v/v)). After this wash with hexane, it is recommended a second wash only with water to assure that there is no hexane left.
3. Afterwards, cells could be resuspended in the minimum water or could be also lyophilized directly.
4. Biomass has to be frozen at (−80°C, overnight) before freeze-drying.

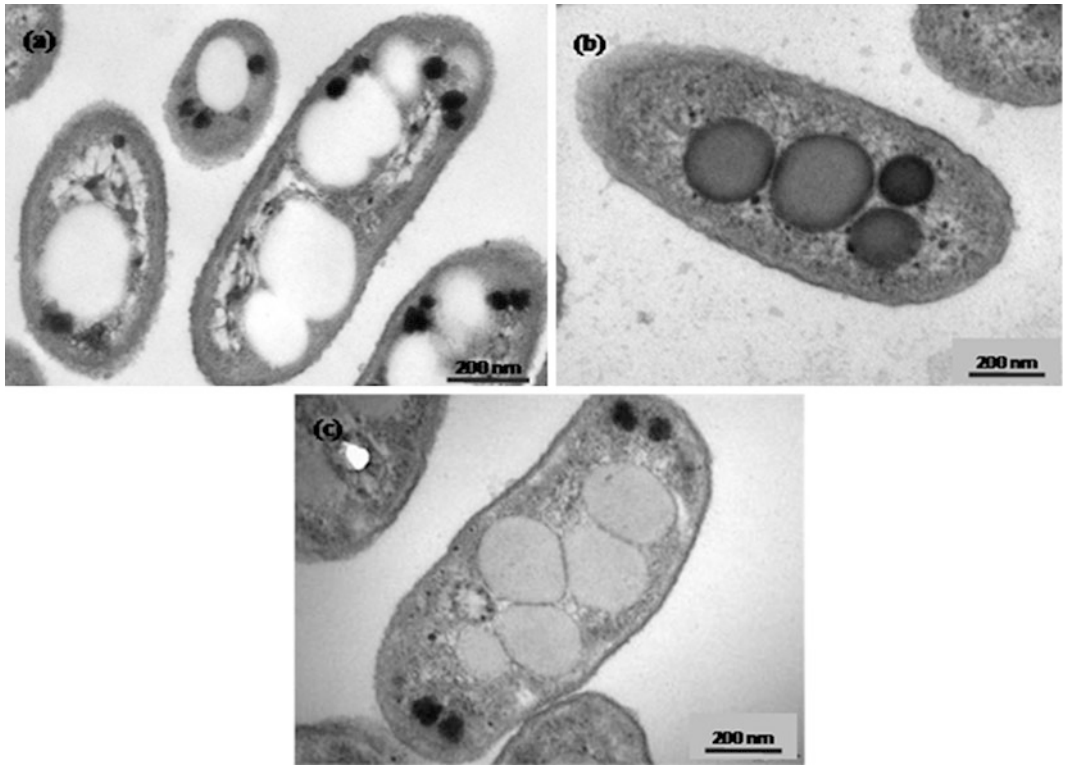

Fig. 3 TEM micrographies. *P. aeruginosa* 42A2 cells grown in minimum mineral medium (MM1) and different substrates: (**a**) Weichol (80% oleic acid), (**b**) undecenoic acid, and (**c**) linseed oil (60% linolenic acid and 20% linoleic acid). The images were acquired in a JEOL JEM 1010 microscope with accelerating voltage of 80 kV ((**a**) Rodriguez [21]; (**b**), (**c**) Bassas [22], with permission))

3.2.2 Polymer Extraction

The classical method of extraction consisted in using chlorinated solvent (e.g., methylene chloride (DCM) or chloroform) and temperature (80°C). Recent publications have shown that hexane or ethyl acetate can be used instead of the chlorinated solvents and that the extraction can be performed at room temperature with agitation [5].

1. The lyophilized biomass of 5–10 mL culture is extracted with 10 mL methylene chloride (DCM) in a Pyrex tube and heated for 3 h at 80°C. Nevertheless, recent publications have shown that when DCM is used as a solvent, its extraction can be carried out at room temperature.
2. The DCM solution must be filtered to remove any cell debris and concentrated by rotary evaporation.

When a relative big amount of biomass has to be extracted, it is useful to use a Soxhlet apparatus. Lyophilized biomass (approx. 2 g in 200 mL of solvent) can also be extracted in a Soxhlet apparatus for 3.5 h [23]. The extract is cooled down to room temperature and solvent removed with a rotary evaporator. This polymer solution

contains mainly the polymer but also small amounts of impurities; therefore a further purification is recommended (*see* Sect. 3.2.3).

3.2.3 Polymer Purification

1. PHA is purified by precipitation from the polymer solution added dropwise into a cold methanol (−20°C) under agitation [23].
2. After polymer precipitation, the solution is kept at 4°C for 1 h. A ratio of organic solvent/MeOH approximately 1/10 (v/v) is recommended.
3. The methanol solution is decanted and the precipitated polymer washed with fresh cold methanol and finally dried in vacuum at 30°C and 30 mbar.

In case that the extracted polymer is going to be used for biomedical applications, it will be necessary a second purification using activated charcoal in order to remove/reduce the endotoxin content that these type of polymers have associated when they are synthetized by a gram-negative bacteria [24].

It is strongly recommended to keep the purified polymer under N_2 atmosphere and at −20°C to prevent further oxidation.

3.2.4 Hydrolysis

It has been described several methods for hydrolyzing the polymer. The most common method mainly consists in simple acidic hydrolysis (methanolysis or propanolysis). In the following subsections, different hydrolytic methods are described. The following protocols of hydrolysis also permit the quantification of the monomer composition of the polymer.

Methanolysis with H_2SO_4/MeOH

The methyl esters were prepared by methanolysis, reacting:

1. 6.5 mg PHA purified is weight in a 10 mL screw-cap Pyrex tube.
2. Addition of 2.7 mL of chloroform, 2.3 mL of methanol, and 0.4 mL of sulfuric acid and heating at 80–100°C for 2 h.
3. After the 2 h of reaction, the mixture is cooled down to room temperature and 1.35 mL of water is added. The mixture is gently vortexed.
4. The water phase is twice extracted with chloroform and all the organic phase is recovered and dried over anhydrous sodium sulfate [25, 26]. The organic extract contains the methyl ester derivatives that could be directly analyzed by gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS).

Methanolysis with H_2SO_4/MeOH and Polymer Quantification

Quantification and analysis of PHA composition are performed essentially as described by Lageveen et al. [18].

1. Lyophilized cells (approximately 15 mg) or purified PHA (approximately 5 mg) are dissolved in 2 mL of 15% (v/v) sulfuric acid in methanol solution.

Note: the amount of lyophilized cells used in the analysis depends on the percentage of PHA accumulation.

2. Two milliliter of chloroform containing 0.5 mg/mL of methyl benzoate (internal standard) is added and the mixture is heated for 140 min at 100°C in a closed screw-cap Pyrex tube.
3. After the time of reaction, the sample is cooled on ice, and 1 mL of demineralized water is added. After vortexing for 1 min, the phases were separated by centrifugation for 5 min at 3,500×*g*.
4. The organic phase is collected, dried over Na_2SO_4, and analyzed by gas chromatography (GC).

Methanolysis with BF_3/MeOH

The methyl ester derivatives could also be obtained using boron trifluoride (BF3) as catalyst in methanol.

1. About 10 mg of purified polymer (20–40 mg of lyophilized cells, depending on the percentage of PHA accumulation) is transferred into a 10 mL screw-cap Pyrex tube.
2. One milliliter of methylene chloride (DCM) containing the adequate internal standard (in a similar concentration as the polymer).
3. After polymer is completely dissolved, 1 mL of BF_3/MeOH solution is added.
4. The tube is kept at 80°C for 20 h. Subsequently, it was cooled to room temperature and 2 mL of saturated NaCl solution for a complete removal of the catalyst is added.
5. The mixture is vigorously shaken, and after phase separation, the aqueous phase was removed. The organic phase is dried and neutralized over a mixture of Na_2SO_4 and Na_2CO_3. The organic phase contains the methyl ester derivatives [27].

Propanolysis with HCl/PrOH

The hydrolysis and transesterification of PHA polymer with propanol and hydrochloric acid were first developed by Riis and Mai for polyhydroxybutyrate analysis (PHB) and later on adapted for PHA analysis [27, 28].

1. About 10 mg of polymer (20–40 mg of lyophilized cells, depending on the percentage of PHA accumulation) is transferred into a 10 mL screw-cap Pyrex tube.
2. One milliliter of methylene chloride (DCM) containing the adequate internal standard (e.g., benzoic acid, methyl benzoate, or 2-ethyl-2-hydroxybutyric acid), in a similar concentration as the polymer).
3. The mixture is kept at room temperature for 1 h until it is completely dissolved.

4. One milliliter of a 20/80 (v/v) mixture of HCl (37%) and n-propanol is added. The mixture is gently shaken and placed at 80°C for 16 h.
5. The organic phase is dried and neutralized over a mixture of Na_2SO_4 and Na_2CO_3. The organic phase contains the propyl ester derivatives ready to be analyzed by GC or GC/MS.

3.2.5 Polymer Derivatization (see **Note 5**)

After being hydrolyzed and methylated (or propylated), the samples could be directly injected and analysis by GC/MS. Sometimes a further derivatization is recommended in order to identify the monomers structure. PHA is mainly constituted for 3-hydroxy monomers that after being converted in the correspondent 3-trimethylsilyl methyl ester (TMSI) derivatives could be easily characterized. The TMSI derivatives are formed by reacting:

1. 5 mg of sample is dissolved in 1 mL of pyridine.
2. Addition of 0.2 mL of hexamethyldisilazane and 0.1 mL of trimethylchlorosilane.
3. The mixture was stirred for 2 h at room temperature.
4. After 2 h of reaction, 5 mL of hexane and 5 mL of water are added.
5. The aqueous layer was then extracted with several volumes of hexane and the organic phase was dried on anhydrous sodium sulfate.

3.3 Characterization of PHAs

In general, the techniques used for the characterization and quantification of these polymers are similar to the ones used for lipid analyses. In the following sections some of the most representative and popular techniques applied to study the structure and the properties of these polyesters will be described.

3.3.1 Structural Techniques

In this section of the chapter, the most commonly used techniques for PHAs characterization are shown. Obviously, in each technique general methods and conditions are described. Therefore depending on the sample and the characteristics of the instrument used, it is possible that some modifications have to be done.

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) is a very useful tool to characterize unsaturated, branched, or functionalized PHAs. The two-dimensional (2D) homonuclear and/or heteronuclear techniques permit the elucidation of double-bond configurations (*cis/trans*) as well as determine whether PHA consists of homopolymer or a copolymer. NMR allows the study of the complete polymer before being hydrolyzed [23, 25, 29–41].

In Figs. 4 and 5 some examples of (1D)-NMR spectra of PHAs are shown.

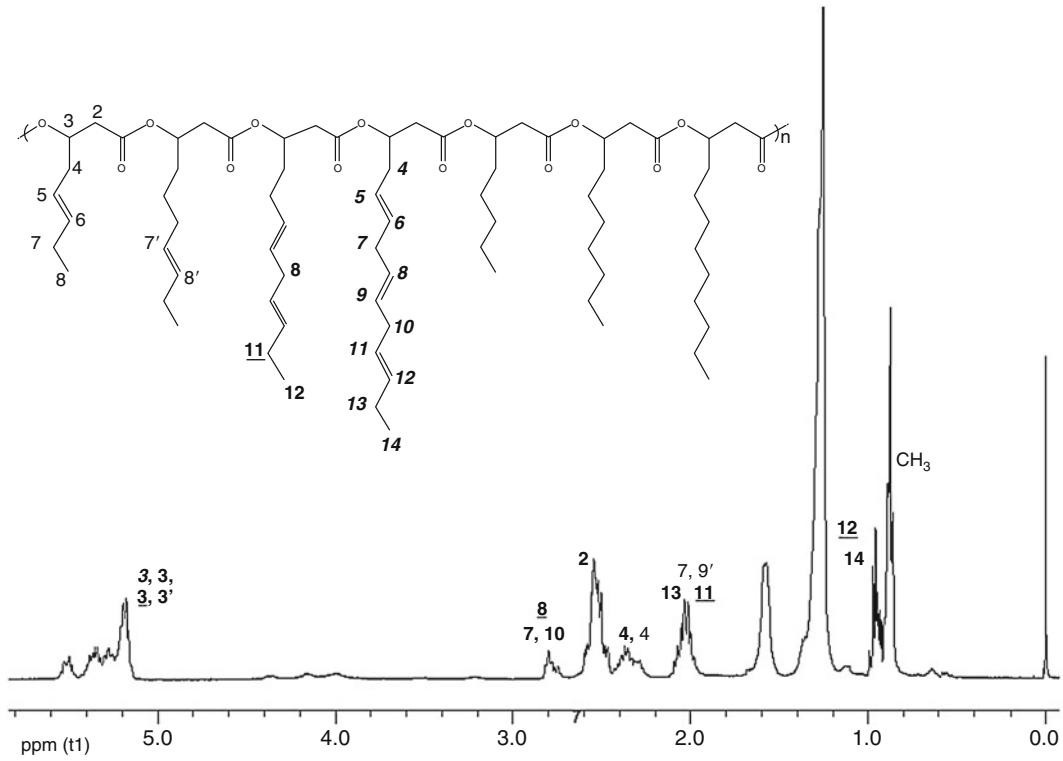

Fig. 4 ^{1}H-NMR spectrum registered in a Mercury 400 (400 MHz). Together with the ^{13}C-NMR spectrum data, the analysis shows the existence of the following monomers: $C_{8:0}$, $C_{8:1}$, $C_{10:0}$, $C_{10:1}$, $C_{12:0}$, $C_{12:1}$, $C_{12:2}$, $C_{14:0}$, $C_{14:1}$, $C_{14:2}$, $C_{14:3}$

The ^{1}H and ^{13}C spectra were recorded in $CDCl_3$ with TMS as internal standard. ^{1}H NMR spectra were recorded in a Varian Gemini 300 (300 MHz) and Mercury 400 (400 MHz). ^{13}C MNR spectra were recorded using the same instruments at 75 and 100 MHz, respectively. Chemical shifts are given in ppm relative to the signal of tetramethylsilane (^{1}H-NMR, 7.26 ppm; ^{13}C-NMR, 77.7 ppm). NMR spectra were recorded in $CDCl_3$ with TMS as internal standard.

For ^{1}H NMR, 5–10 mg of polymer was dissolved into 0.7 mL of $CDCl_3$ and 10–20 mg of polymer is needed for the ^{13}C spectra. The ^{1}H NMR spectra in the Gemini 300 were recorded with a pulse width of 8 μs (45° pulse), a spectral width of 4,500 Hz, an acquisition time of 3 s, and a relaxation time of 1 s, with 64–128 scans for the required signal-to-noise ratio.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC/MS analysis is a suitable method to understand and determine PHAs structure. This technique is especially useful in the case of long-chain-length monomers that could not be identify by

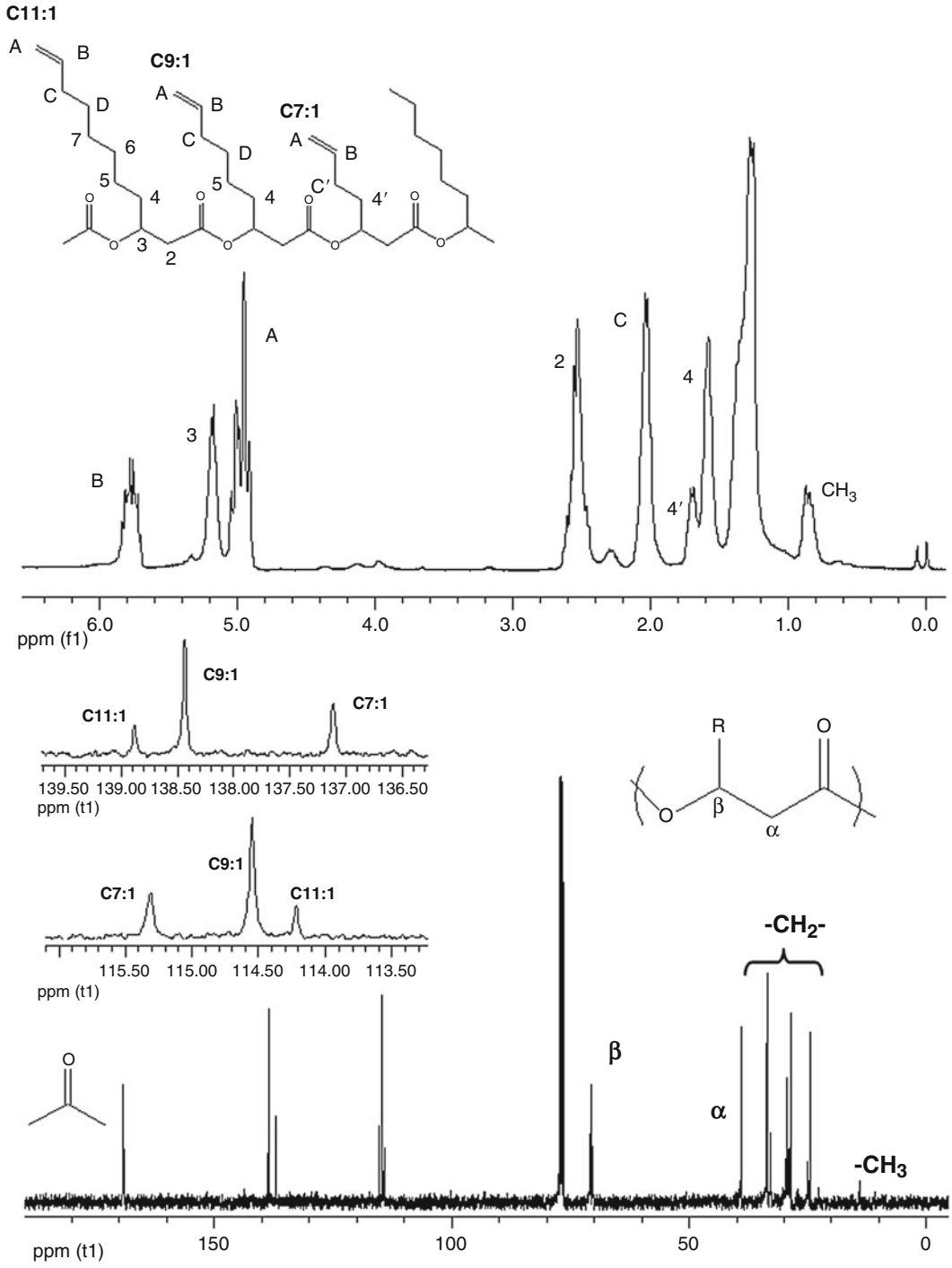

Fig. 5 ^{1}H and ^{13}C NMR spectra of PHA-C11:1 obtained from a culture of *P. aeruginosa* 42A2 using undecenoic (1% (v/v)) acid as a carbon source. The spectra are recorded in a Gemini 300 (300 MHz)

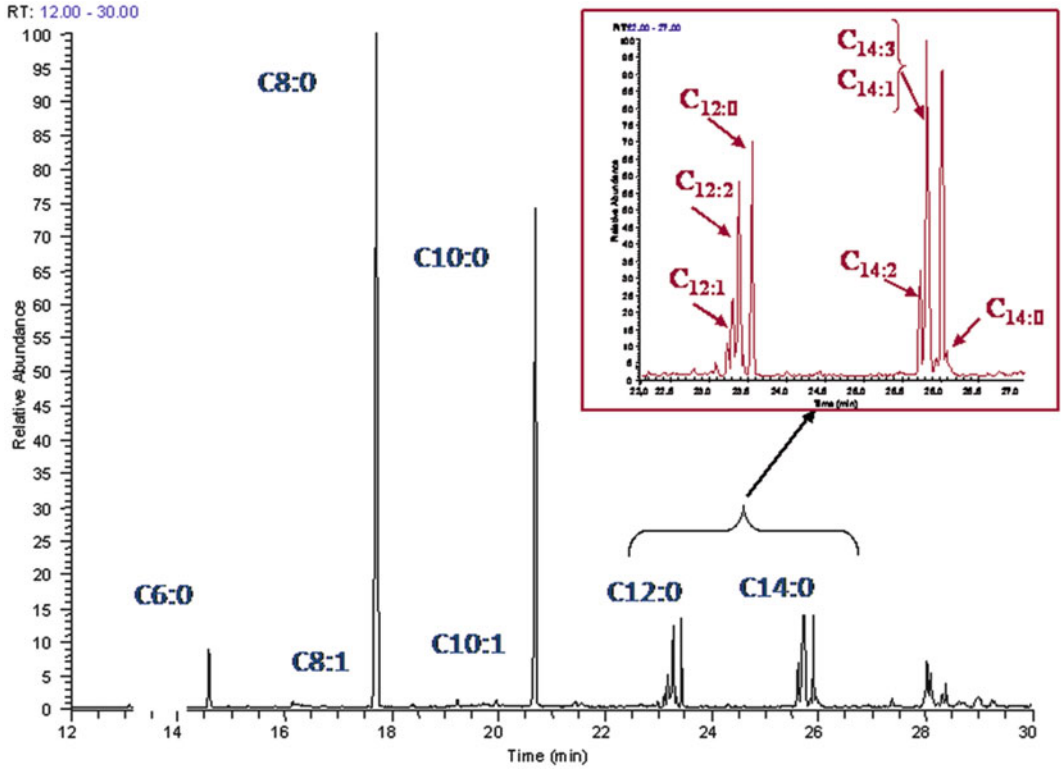

Fig. 6 GC analysis. Chromatogram of PHA obtained from a culture of *P. aeruginosa* 42A2 grown in minimum mineral (MM1) and using linseed oil as a carbon source

^{13}C-NMR due to the similar carbon atom chemical shifts or by ^{1}H-NMR spectra patterns which are too complicated to be used for a structural analysis. However, GC is only useful for volatile compounds. Thus, some chemical derivatizations are required. PHAs need to be hydrolyzed, acylated, and sometimes further chemically derivatized (*see* Sects. 3.2.4 and 3.2.5). In Figs. 6 and 7 the chromatogram and the mass spectra, respectively, of a polyunsaturated PHA are shown, as an example.

GC/MS analysis is carried out with a trace DSQ instrument equipped with a HP5 column (cross-linked 5% Phe silicone, Hewlett-Packard) (25 m × 0.20 mm i.d. × 050 μm) and a TRACE-DSQ mass spectrometry detector (Thermo Finnigan, Waltham, MA, USA), set to scan from m/z 60 to 700 a.m.u. The oven temperature is programmed from 35 (2 min) to 310°C (10 min) at 8°C/min. The injector temperature is 250°C in split injection mode (1:30) and the detector transfer line was at 280°C. One microliter of TMSI derivative solution (prepared according Sect. 3.2.5) is injected. The molecular weight of the monomers is determined by chemical ionization using methane as

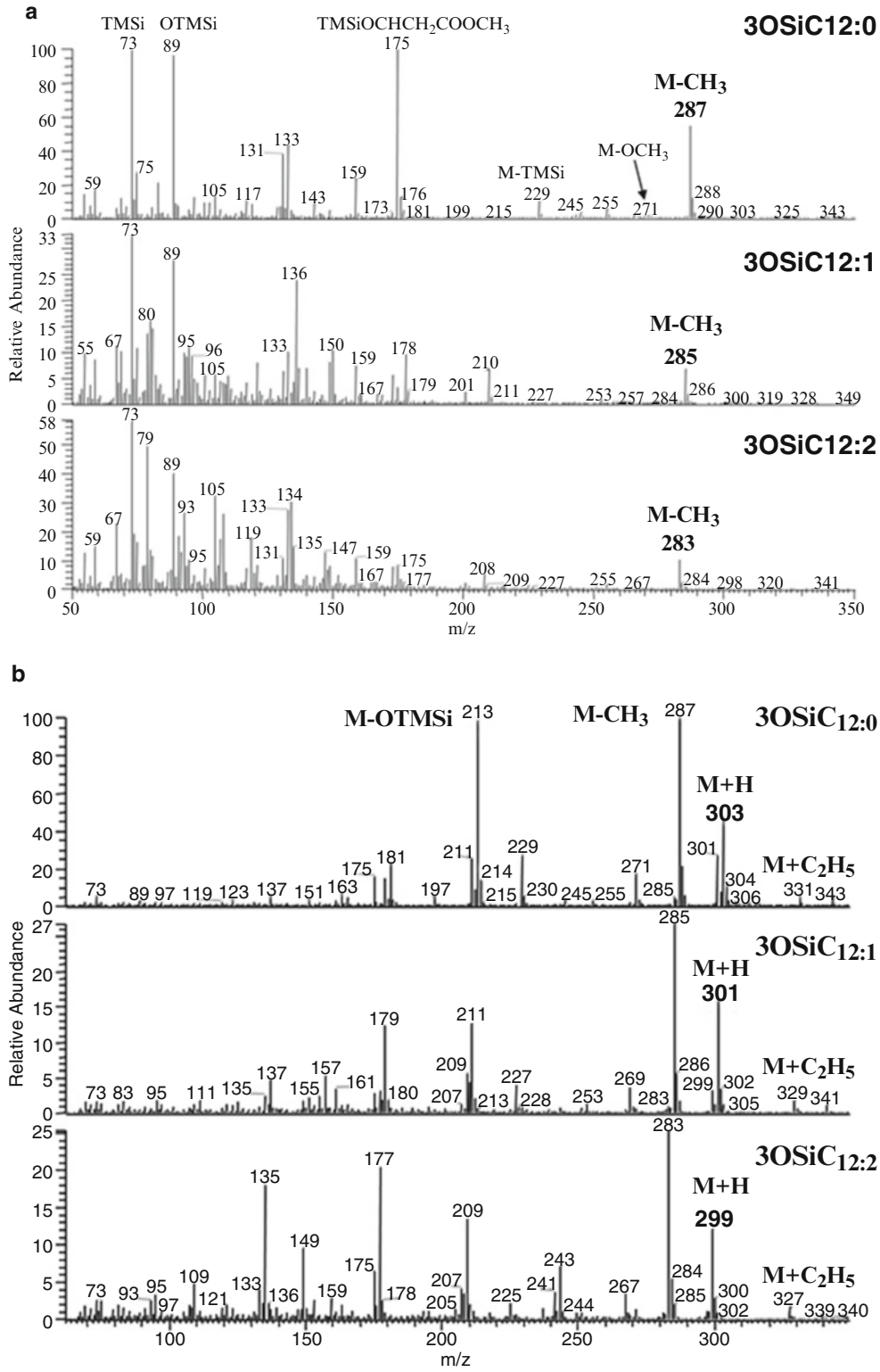

Fig. 7 GC/MS analysis: (**a**) electron impact pattern fragmentation of the family of 3OH-C12 (3OHC12:0, 3OHC12:1, and 3OHC12:2)-3 and (**b**) its chemical ionization (CH_4) mass spectra

the ionizing gas. Structural information is obtained by electron impact (70 eV). Helium is used as the carrier gas at a flow rate of 1 mL/min [28, 42, 43]. In Fig. 6 an example is shown. The analysis corresponds to a PHA produced using linseed oil as a carbon source. As it could be observed in the chromatogram, the polymer is mainly constituted by 3-hydroxy-octanoate (3OH-C8) and 3-hydroxydecanoate (3OH-C10), although other 3-hydroxy monomers were found.

The GCMS spectra of the TMS derivatives from saturated and unsaturated 3-hydroxyalkanoic acid methyl esters exhibited characteristic fragments at m/e 73 $\{(CH_3)_3Si^+\}$, m/e 89 $\{(CH_3)_3SiO^+\}$, m/e 131 $(C_5H_{11}SiO_2)^+$, m/e 133 $(C_5H_{13}SiO_2)^+$, and m/e 159 $(C_6H_{11}SiO_3)^+$. Because of the α-cleavage to the derivatized hydroxyl group, two more characteristic fragmentations were also observed: m/e 175 $\{(CH_3)_3Si^+ = CHCH_2CO_2CH_3\}$ and [M-73] $(RCH = O^+Si(CH_3)_3)$. All of them are known to be characteristic peaks for the TMSI derivatives of 3-hydroxyl functional groups [44]. The chain lengths of the TMSI derivatives of 3-hydroxyalkanoic acid methyl esters can be determined from the molecular weight of the molecular ion-related m/e fragment [M-15]. The existence of a double bond can be deduced from the molecular weight of the m/e fragment [M-15] from the unsaturated monomer, which has two units less than that of the corresponding saturated monomer. The molecular weight was also confirmed by examining the chemical ionization (CH_4-CI) mass spectra (*see* Fig. 7).

The profile of mass spectra for the methyl esters of saturated 3-hydroxyalkanoic acids is characterized for a predominant peak at m/z = 103 that corresponds to the α-cleavage between C3 and C4. Another two characteristic peaks for these compounds are: m/z = 74 (due to a McLafferty rearrangement of the methyl ester) and m/z = 71 (as a consequence of the loss of a methanol molecule from the peak m/z = 103) [44, 45].

3.3.2 Thermal Properties: DSC and TGA

The thermal properties of the microbial polyesters are determined by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA), using 10 mg of the purified polymer in each analysis. DSCs are performed on a DSC-30 and TGAs in a thermobalance TGA-SDTA 851e/SF/1100 (Mettler-Toledo Instruments, NY, USA). In the DSC analyses, samples are placed on an aluminum pan and heated from −100 to 400°C at 10°C/min under air or N_2 (80 mL/min). In the TGA analyses, samples are processed at a rate of 10°C/min from room temperature to 700°C under air or N_2 (50 mL/min). All data were acquired using STARe System acquisition and processing software (Mettler-Toledo) (*see* **Note** 6).

3.3.3 Molecular Weight Distribution: Gel Permeation Chromatography

Molecular weight distribution (MWD) could be determined by gel permeation chromatography (GPC). The analysis conditions depend on the instrument and column used. Standard analysis conditions are described below:

Molecular weights (number average (Mn) and weight average (Mw)) are determined using a Waters Styragel 5E column (Waters, Mildford, MA, USA) with a Knauer refractive index detector. The detector is set at 47°C and 2.0 bar. Tetrahydrofuran (THF) is used as an eluent at a flow rate of 0.5 mL/min and 40°C. Fifty microliters of a 0.4 mg/mL solution of purified PHA in THF is injected. The calibration curve is created with polystyrene standards of low polydispersity (Agilent Standard Kit, www.chem.agilent.com) with molecular weight (Mw) range of 580–3,000,000 Da. The standard concentration depends on the column requirements (e.g., 0.2 mg/mL).

4 Notes

1. Especially in the SEM and TEM sample preparation, some very specific equipment (*see* steps 9, 10, and 11) and reagents are needed. Therefore it is highly recommended to perform the sample preparation in a specialized labs or outsource the samples to specific microscopy facilities.
2. The volume of sample should be adjusted considering the optical density of the culture.
3. The volume of the sample will depend on the optical density of the culture. It is also possible to fix the samples by using a glutaraldehyde (4%) solution in phosphate buffer (PB). In addition, note that different buffer solutions and coatings can be used.
4. The duration of the process will depend on the amount of biomass and its water content as well as of the equipment used.
5. This reaction must be carried out in completely anhydrous conditions and under the fume hood. In these conditions, the 3-trimethylsilyl methyl esters obtained are dissolved into 350 μL of chloroform for GC-MS analysis, but final volume should be adjusted according the final concentration and the equipment requirements.
6. This section is showing standard analysis conditions for PHA analysis. Obviously, the thermal properties depend on the polymer composition; therefore easily some parameters in the analysis might need to be adjusted.

5 Research Needs

The chapter shows the main guidelines for the recovery and analysis of polyhydroxyalkanoates. Most of the procedures can be applied in the analysis of other storage compounds with the adequate

modifications. All the analytical methods described are standard analytical techniques that have been adapted considering the new needs. Neither of them can be used as an absolute tool, especially in the monomer composition analysis. It has been shown that they are complementary tools that contribute to the final elucidation of the structure. Despite these techniques that have undergone important development in the last decades, it is still necessary to enhance the rapidity, specificity, and accuracy of these methods. In the future, next steps should be focus to obtain faster and precise analytical methods together with faster and cheaper downstream processes to keep costs low (energy costs), especially in large-scale processes

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Purification of Lipid Rafts from Bacterial Membranes

Charlotte Wermser and Daniel Lopez

Abstract

The membranes of eukaryotic cells contain microdomains that are different in lipid composition to the surrounding membrane and aggregate a number of proteins related to signal transduction and protein trafficking. These are referred to as *lipid rafts* or *membrane rafts* and are specialized in the regulation of cellular processes related to signal transduction, protein sorting, and membrane trafficking. The integrity of lipid rafts is important for the correct functionality of these raft-harbored cellular processes, and their alteration is related to the occurrence of severe diseases. We recently discovered that the membranes of bacterial cells also organize their signal transduction pathways in functional membrane microdomains that are structurally and functionally similar to the lipid rafts of eukaryotic cells. The existence of lipid rafts in the membrane of bacteria suggests that bacteria are more complex organisms than previously appreciated, and thus, their cellular complexity should be explored in more detail. In this protocol, we provide a detailed description of the materials and techniques that are necessary to purify the lipid rafts from bacterial membranes, which is a necessary step to explore the number of proteins and lipid species that constitute these membrane platforms. This is an essential protocol for any laboratory interested in exploring any aspect related to organization of lipid rafts in bacterial membranes.

Keywords: *Bacillus subtilis*, Bacteria, Flotillin, Lipid rafts

1 Introduction

Compartmentalization of cellular processes in organelles improves their efficiency and the specificity of biological reactions. Physical compartmentalization of proteins in subcellular domains increases proximity and the probability of the assembly of specific protein complexes and signaling cascades (1). Because of this, there are a number of cellular compartments that specialize in specific cellular processes (2). For instance, mitochondria are specialized in respiration and generation of energy. The endoplasmic reticulum is functionally specialized in the translation of proteins (2). However, possibly one of the most recent examples of cellular compartmentalization is the aggregation of a large number of membrane proteins related to signal transduction and protein trafficking into membrane microdomains that are different in lipid composition to

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the surrounding membrane (3). These membrane domains are referred to as *membrane rafts* or *lipid rafts*, and they are responsible for the organization and functionality of many diverse cellular processes related to signal transduction and protein trafficking (3–5).

The organization of the cellular membrane into membrane microdomains or lipid rafts is a relatively recent concept in biology. The first membrane model proposed by Sanger and Nicolson suggests that lipid and protein components of cellular membranes diffuse freely and randomly (hereof, its name of "fluid mosaic model") (6). Therefore, it was assumed that all protein and lipid constituents were homogeneously distributed across the biological membrane. However, the pioneering fluid mosaic model has been subjected to several interpretations in the past decades. Specifically, it was discovered that biological membranes are composed of several distinct lipid species that tend to coalesce in specific areas simply due to their physicochemical affinities (4, 7). Importantly, the heterogeneous organization of distinct lipids into discrete membrane microdomains leads to a diverse distribution of the proteins. The particular subcellular distribution of proteins appears essential for their functionality (7–9). The lateral organization of lipids and proteins in specific regions of the membrane is nowadays referred to as membrane domains (10, 11).

Many types of cells organize membrane domains that are specialized in regulating diverse cellular processes. For example, polarized epithelial cells show basolateral and apical membrane macrodomains that contain a different composition of lipids and proteins and are specialized in different biological functions (12–14). Neurons also show different membrane domains exhibiting particular lipid and protein composition, according to their role in synapses (15, 16). The membranes of eukaryotic cells also organize a variety of proteins related to signal transduction and membrane trafficking into the lipid rafts. Those are discrete membrane regions at the nanoscale level that are enriched in specific lipids, like cholesterol or sphingolipids (3). Lipid rafts are well-organized membrane structures whose organization requires the activity of specific proteins that are known to exclusively localize within lipid rafts. One of these raft-associated proteins is flotillin (17–21). Flotillin is a membrane chaperone that acts as a scaffolding protein to facilitate the recruitment of the proteins that need to localize in lipid rafts to be active and facilitates their interaction (17–21).

Initially, the existence of lipid rafts was exclusively associated with eukaryotic cells because prokaryotic cells are unable to produce cholesterol and, therefore, unable to organize cholesterol-rich membrane microdomains (3–5). In addition to this, bacteria were considered too simple organisms to require a complex compartmentalization of their membranes and signaling networks. Consequently, it was unlikely that bacterial cells were able to assemble lipid rafts in their membranes. However, recent subcellular compartmentalization

structures have been found in bacteria (22), including the discovery that bacterial cells are able to assemble membrane platforms that are functionally and structurally similar to the lipid rafts of eukaryotic cells (23). Bacterial lipid rafts are formed by the self-aggregation of noncyclic polyisoprenoid lipids and their co-localization with raft-associated flotillin proteins (23–27). Similarly to eukaryotic flotillins, bacterial flotillins seem to act as scaffold proteins to recruit the protein cargo of the lipid rafts and facilitate the interaction and oligomerization (23–27). Bacterial lipid rafts were discovered in the model organism *Bacillus subtilis*, but it is believed that many other bacterial species are able to assemble lipid rafts in their membranes.

As many prokaryotes lack cholesterol in their membranes (28), the functional organization of the membrane into microdomains or lipid rafts depends on the existence of lipid species different from cholesterol. The exact nature of these has not yet been shown, but there is experimental evidence that other polyisoprenoid lipids that may have similar physicochemical properties to cholesterol play a role in the assembly of lipid rafts in bacteria (28, 29). For instance, *B. subtilis* produces membrane-associated polyisoprenoid lipids of carotenoid nature (30, 31). When the synthesis of carotenoids is inhibited in *B. subtilis* cells, the integrity of the microdomains is perturbed (23). Importantly, the integrity of the microdomains can be reconstituted by adding carotenoids to the cultures of the deficient *B. subtilis* strain (23). The integrity of the bacterial functional membrane microdomains also depends on the activity of the raft-associated protein flotillin. This protein is well conserved in all kingdoms of life and is indeed expressed in many bacterial species (32). It is a scaffold protein that facilitates the recruitment and interaction of the raft-associated proteins (17–21).

In summary, regardless of the species under consideration, lipid rafts could be described as rigid, compact, and superhydrophobic membrane microdomains that concentrate a specific composition of lipid and proteins and are specialized in regulating certain cellular processes (3–5). One of the key aspects regarding the research of lipid rafts has been the development of reliable techniques to allow the purification and further examination of lipid rafts. The most reliable purification technique is based on the ability of the lipid rafts to resist disaggregation when the membrane fraction is treated with a mixture of nonionic detergents. This treatment generates one membrane fraction that is sensitive to detergent disaggregation (detergent-sensitive membrane fraction, DSM) and another fraction that is composed of larger membrane fragments because they were more resistant to detergent disruption (detergent-resistant membrane fraction, DRM) (33). These two different membrane fractions can be physically separated according to their size in a sucrose gradient and analyzed independently. It is important to emphasize that the DRM fraction should not be equated to the lipid raft fraction, although it is known that this fraction is highly

enriched in lipid rafts (33). However, one should always consider that this is an artificial technique for lipid raft purification that could generate biased results, causing proteins to migrate to one fraction or another due to their efficiency to bind the detergent, their size, the temperature during the separation process, or the time that the separation took place (4, 5). In fact, the non-rigorous use of this methodology in the past led to the generation of data difficult to reconcile and opened a debate about the existence of lipid rafts and whether lipid rafts were artifacts generated during the purification process (34, 35). Therefore, the purification of the DRM fraction is an excellent point to start the analysis of lipids and proteins that constitute lipid rafts, but further experiments need to be performed to validate any potential lipid and protein candidate. Biochemical procedures based on protein-protein interaction assays using pull-down assays or a bacterial two-hybrid system are generally preferred to address further questions related to raft-associated lipids and proteins.

We present in this section a detailed methodology for isolating the DRM fraction from the cellular membrane of the bacterial model *B. subtilis*. This methodology can be applied to other bacterial models with minor modifications. This protocol for the purification of the DRM fraction should be of interest to any laboratory interested in the study of bacterial lipid rafts (Fig. 1).

2 Materials

2.1 Cell Growth, Harvesting, and Lysis

1. Growth medium: Luria-Bertani medium (LB) 0.5% NaCl, 1% tryptone, 0.5% yeast extract, pH 7. To prepare solid media, bacto-agar at the final concentration of 1.5% was added to the final growth medium.
2. Lysozyme solution 1 mg/ml in PBS buffer (Roth).
3. Phenylmethanesulfonyl fluoride (PMSF) solution 100 μM in DMSO (AppliChem).
4. DNase I (New England Biolabs®).
5. Cell lysis device: French press (10,000 psi, 4 passes) (SLM Aminco Instruments).

2.2 Membrane Purification

1. Buffer H (see general buffers)
2. Purification device: Ultracentrifuge Optima™ L-80 XP (Beckman Coulter) (100,000 × *g*)
   Rotor 70.1 Ti (fixed angle) with appropriate polycarbonate tubes
3. Glycerol 10% solution in buffer H

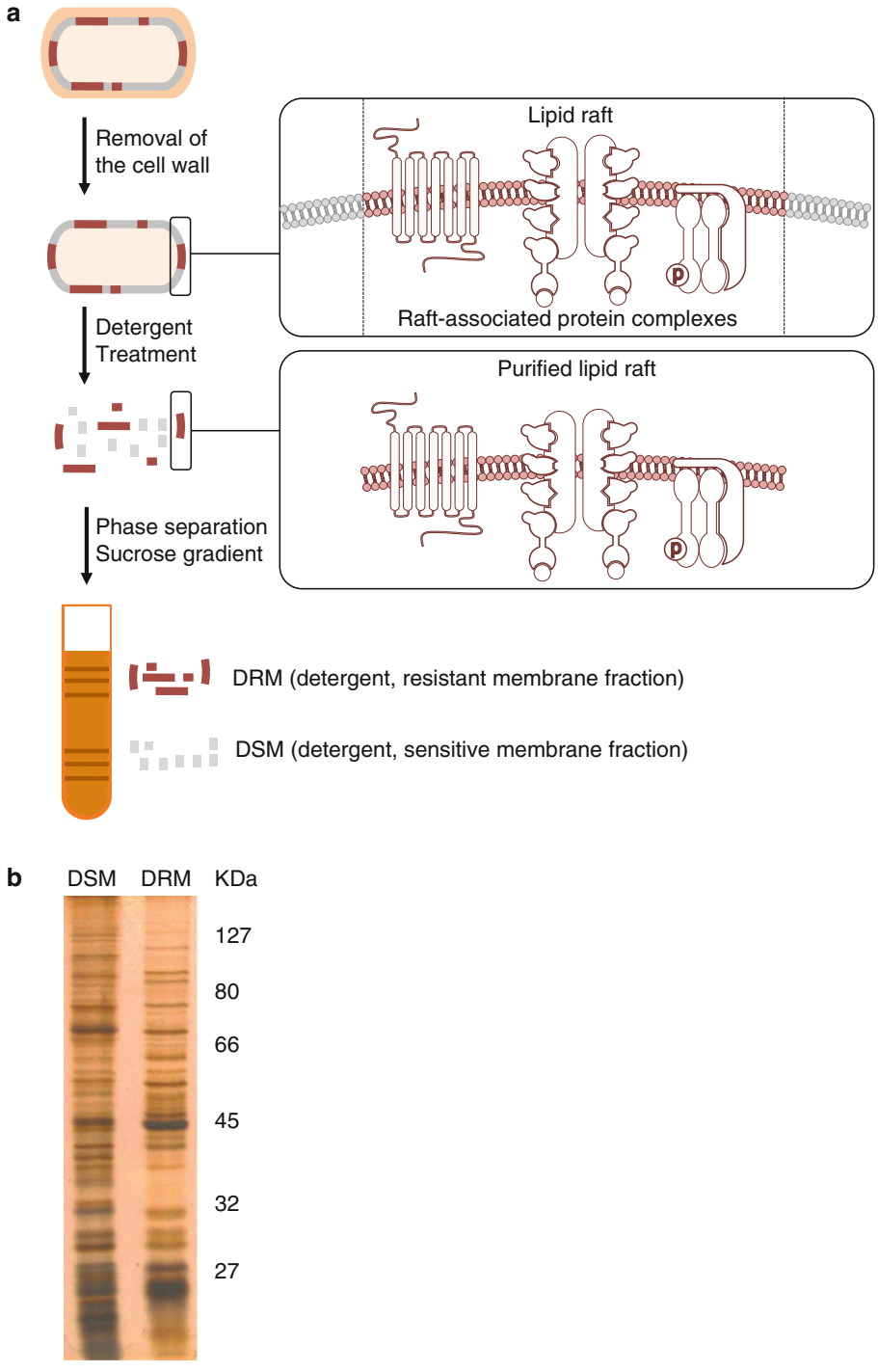

Fig. 1 Purification of the DRM fraction from *B. subtilis* membranes. (**a**) Schematic representation of the protocol for the purification of the DRM fraction of *B. subtilis* membranes. This approach could be applied to other bacterial species. After cell wall removal using a lysozyme treatment, the membrane fraction is disaggregated using a mixture of nonionic detergents. Lipid rafts are resistant to detergent disruption and

2.3 Membrane Disaggregation

1. Detergent cocktail: Triton X-100, Lubrol, Brij 96, Nonidet, CHAPS, and octylglucoside (concentrations from 1% to 4%) (all from Sigma-Aldrich).
2. Buffer H.

2.4 Sucrose Gradients

1. Eighty percent sucrose solution in 0.2 M sodium carbonate.
2. Twenty percent sucrose solution in buffer H.
3. Sucrose gradient device 1: Ultracentrifuge (40,000 × *g*)
   Rotor SW40 Ti (swinging bucket) with appropriate polycarbonate tubes
4. Optional: Sucrose gradient maker

2.5 Fraction Examination

1. Ice-cold acetone.
2. Buffer PBS.
3. 1× Protein loading buffer: 62 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue.

2.6 Bacteria Strain

1. *Bacillus subtilis* strain 168 was used a reference strain.

2.7 General Buffers and Reagents

1. Buffer PBS = 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7
2. Buffer *H* = 20 mM HEPES, pH 8, 20 mM NaCl, 1 mM DTT, 1 mM PMSF

3 Methods

3.1 Cell Growth, Harvesting, and Cell Lysis

We provide in this protocol the general procedure to grow bacterial cells in liquid medium. Alternatively it is also possible to collect cells from solid medium. Growth conditions could change depending on the species under consideration.

1. Grow *B. subtilis* strain 168 in 100 ml of LB liquid medium at 37°C for 24 h. It is important to allow cells to reach stationary phase before harvesting (*see* **Note 1**).

Fig. 1 (continued) remain larger fragments that can be further separated from the rest of the membrane by zonal centrifugation using a sucrose gradient. Zonal centrifugation renders two different membrane fractions: a DSM fraction (detergent-sensitive membrane fraction) that is composed of smaller fragments and is the consequence of a successful disruption by detergent treatment and another detergent-resistant membrane fraction (DRM) that is composed of larger fragments because they were not disrupted by the detergent treatment and is supposed to be enriched in lipid rafts. (**b**) Analysis of the protein composition of the DRM and the DSM fractions using SDS-PAGE. The electrophoresis analysis shows that there is a different protein composition in the DSM and the DRM fraction, suggesting that the subcellular localization of membrane proteins is strongly influenced by the different lipid composition of the cellular membrane

2. Pellet the cells by centrifugation (10 min, 4,000 rpm, 4°C) and remove the supernatant. Wash the cells twice with PBS buffer. At this point, the washed dry pellet can be stored at –20°C until further usage.
3. Resuspend the cell pellet in 10 ml of buffer H. It is important to ensure that cells are well resuspended and dispersed in buffer H. The presence of cell clumps will prevent a correct cell lysis.
4. To lyse the cells, add 200 µl of lysozyme (1 mg/ml), 100 µl of PMSF (100 µM), and 5 µl of DNase I to the suspension and incubate the sample at room temperature for 30 min. This step is necessary to dissolve the peptidoglycan cell wall of bacterial cells, which is especially prominent in gram-positive bacteria. Next, pellet the cells by centrifugation (10 min, 4,000 rpm, 4°C) and carefully resuspend in 10 ml buffer H (*see* **Note 2**).
5. Perform cell disruption using a French press (10,000 psi and 4 passes). After disruption, eliminate cell debris by centrifugation (10 min, 12,000 rpm, 4°C) and use the supernatant for membrane purification.

3.2 Membrane Purification

1. The membrane fraction can be purified using an ultracentrifuge with a fixed angle rotor (100,000 × *g* for 1 h at 4°C).
2. Discard the supernatant and dissolve the membrane fraction in 200 µl buffer H supplemented with glycerol 10% by persistent pipetting (*see* **Note 3**).
3. At this point, the membrane fraction dissolved in 200 µl buffer H + glycerol 10% can be stored at –20°C until further usage. Alternatively, you can immediately proceed with the membrane disaggregation step.

3.3 Membrane Disaggregation

1. Disruption of the cellular membranes should be performed using a mixture of nonionic detergents such as Triton X-100, Lubrol, Brij 96, Nonidet, CHAPS, or octylglucoside (*see* **Note 4**).
2. Use a range of concentration of detergents between 1% and 4% for the disruption of cell membranes. Unfortunately, the optimization of the detergent treatment needs to be determined empirically. The disruptive activity of each detergent component from the mixture depends on external factors like the duration of the procedure or the temperature of the process (*see* **Note 5**).
3. In our particular case, we normally use a mixture of Triton X-100, Brij 96, and Nonidet, 1% for a membrane disruption process of 30 min at 4°C (*see* **Note 6**). We determined these conditions empirically as referred to in Sect. 3.3(2) and **Note 5**.

3.4 Sucrose Gradients

1. Mix the sample thoroughly with 800 µl 80% sucrose in 0.2 M sodium carbonate (keep sample at 4°C).

2. Transfer the sample into an ultracentrifugation tube and carefully overlay it with 4 ml 20% sucrose in buffer H and 3 ml 10% sucrose in buffer H, respectively (keep sample at 4°C) (*see* **Note 7**).
3. Separate the sucrose gradient fractions by ultracentrifugation with a swinging bucket rotor (15 h, 40,000 × g, 4°C).
4. Carefully take fractions of 1 ml volume by aspiration from the very top of the sucrose gradient and keep the samples on ice for further protein precipitation and analysis (*see* **Note 8**).

3.5 Fraction Examination

1. To analyze the protein content of each membrane fraction, protein precipitation can be performed to purify the protein content of the samples. To do this, add 4 volumes of ice-cold acetone to each sample fraction.
2. After 2 h of incubation at −20°C, precipitate denatured proteins by centrifugation (20 min, 15,000 rpm, 4°C).
3. Remove the supernatant and dry the pellets at room temperature for up to 2 h.
4. The protein pellet should be carefully and repeatedly washed with PBS buffer. After washing, resuspend the protein samples in 1× protein loading buffer and store at –20°C prior to protein analysis.
5. For separation of protein samples, use SDS polyacrylamide gels consisting of a stacking gel and a resolving gel with 12% acrylamide/bisacrylamide (37.1:1).
6. Stain the SDS gel with Coomassie to visualize the differential protein contents of the DSM and DRM.

4 Notes

1. The diversity of lipid composition in bacterial membranes is greater in cells undergoing stationary phase because many unusual membrane lipids are produced at stationary phase. Alternatively, cells grown on solid agar medium generally show more variety in the cell growth and allow detecting a more diverse lipid composition in bacterial membranes.
2. The cell pellet needs to be handled with care at this point. The absence of a cell wall makes bacterial cells extremely sensitive to any type of stress.
3. It is important to note that the purified membrane fraction is strongly enriched in membrane lipids and therefore, it is hard to dissolve in aqueous buffers like buffer H. The addition of glycerol 10% to the buffer H is highly convenient to dissolve the membrane fraction in buffer H. However, dissolving the membrane fraction still requires vigorous pipetting.

4. These nonionic detergents are optimal to particularly disaggregate cellular membranes according to their lipid composition. The unusual lipid composition of lipid rafts, which are enriched in noncyclic polyisoprenoid lipids, provides rigid, compact, and hydrophobic properties to these membrane microdomains (like a floating raft, hereof its name of lipid raft), which confer to lipid rafts the capacity to resist detergent disaggregation by nonionic detergents.
5. An alternative approach to purify the DRM can be achieved with the purification kit CelLytic™ MEM for protein extraction (Sigma-Aldrich® Ref. CE0050). The kit contains a mixture of nonionic detergents, which has been optimized to achieve a high-performance purification of the DRM fraction. The kit also contains a gel phase that separates into two fractions in response to lower temperatures (4°C). DSM and DRM membrane fractions separate with the gel phases after treatment and can be further isolated and purified.

   We performed a comparative analysis between the CelLytic MEM kit and the traditional purification method. The protein composition of the DRM fraction was similar using both approaches.
6. When using any of these two technical approaches for membrane phase separation, it is crucial that manipulation of cellular membranes and detergent disruption procedures are performed at 4°C. This is because low temperatures enhance the separation of the different membrane lipids and the stabilization of lipid ordering in bacterial membranes.
7. Distinct layers of different sucrose concentrations must be visible prior to ultracentrifugation. In order to avoid mixing of the different solutions let sucrose slowly run down the wall of the ultracentrifugation tube. Alternatively, a gradient maker can be used to make the sucrose gradients.
8. Fractions of 1 ml each are a good starting point for the analysis of raft-association of a protein. Taking more fractions improves the resolution of the assay, but also increases the necessary time and effort.

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Isolation and Analysis of Membrane Phospholipids as Biomarkers

Nancy Hachicho and Hermann J. Heipieper

Abstract

The protocol describes the analysis and data interpretation of membrane phospholipid fatty acid (PLFA) profiles as biomarkers for the characterisation of microbial communities in environmental samples, with special emphasis on soils and sediments contaminated with organic hydrocarbons. This simple method in microbial ecology studies provides an estimate of the viable microbial biomass, first indications of the structure of the microbial community, as well as the physiological status of the microbiota. In environmental biotechnological applications, PLFA analysis is a useful tool for monitoring bioremediation and allows direct validation of existing biological activities as well as hints for shifts in the microbiota caused by successful biological treatment strategies.

Keywords: Biodiversity, Biomarkers, Community analysis, Microbial ecology, Phospholipid fatty acids (PLFA), Stress response

1 Introduction

The contamination of soils, sediments and groundwaters with hazardous hydrocarbons such as fuel components represents one of the biggest problems of environments all over the world. Therefore, many attempts have been carried out to remediate such sites, commonly highly polluted with alkanes and BTEX compounds. Among these techniques, bioremediation seems to be the most promising one. However, the direct proof for in situ biological catabolic activity is quite difficult and/or cost intense. So far, bioremediation studies are often restricted to following the disappearance of hazardous pollutants [1, 2] and still regard this system as a black box. As it is known that traditional culture-dependent methods are highly biased when analysing environmental samples [3], culture-independent methods have been applied since two decades in order to characterise microbial community structures and their shifts under changing environmental conditions. One possibility for a culture-independent method to estimate biological

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activity in situ is the use of lipids as biomarkers [4–9]. The use of phospholipid fatty acid (PLFA) analysis in microbial ecology studies provides, with an estimate of the viable microbial biomass, first indications for the structure of the microbial community, as well as the physiological status of the microbiota. Furthermore, the phenotypic response of microorganisms to environmental conditions is reflected by PLFA [10–13]. For environmental monitoring and risk assessment in soils, the PLFAs serve as important bioindicators [14]. The procedure to use PLFAs as biomarkers for the analysis of microbiota, especially in hydrocarbon-contaminated soils and sediments, will be described in the following protocol.

2 Materials

2.1 Solutions and Materials

Only chemicals needed are chloroform, methanol, acetone and hexane. Silica gel columns (Bakerbond spe, Baker) are used for separation of the total lipids into neutral lipids, glycolipids and phospholipids by subsequent elution with chloroform, acetone and methanol. For the methylation, a trimethylchlorosilane (TMCS, Merck, Darmstadt, Germany) solution in methanol (1:9, v/v) or boron trifluoride in methanol (BF_3 MeOH complex, Merck, Darmstadt, Germany) is applied. GC-autosampler flasks (Nalgene, Rochester, USA) are the only special equipment needed; a vortex apparatus and different centrifuges should be present in every routine lab.

2.2 Time Considerations

For the complete extraction of wet soil or sediment, samples to FAME about 2 days should be taken into account, although the actual working times are far less intense than the incubation breaks (lyophilization, phase separation, evaporation steps). An autosampler-equipped GC-FID and GC-MS can analyse the FAME overnight. Data analysis is regularly done by the GC-supporting hard- and software (e.g. ChemStation, Agilent).

3 Methods

3.1 Sampling of Soils and Sediments for Lipid Analysis

Generally, the amount of material necessary for an efficient lipid and fatty acid analysis of soils and sediments is bigger than needed for molecular biological or culture-dependent methods. Of course, the amount of material is strongly depending on the density of biomass in the certain environment. For bacteria, cell amounts of about 10^9 cells need to be taken in order to guarantee a sufficient lipid and fatty acid analysis. The following protocols recommend 2 g of soil material that was previously lyophilised for 24 h before starting the total lipid extraction.

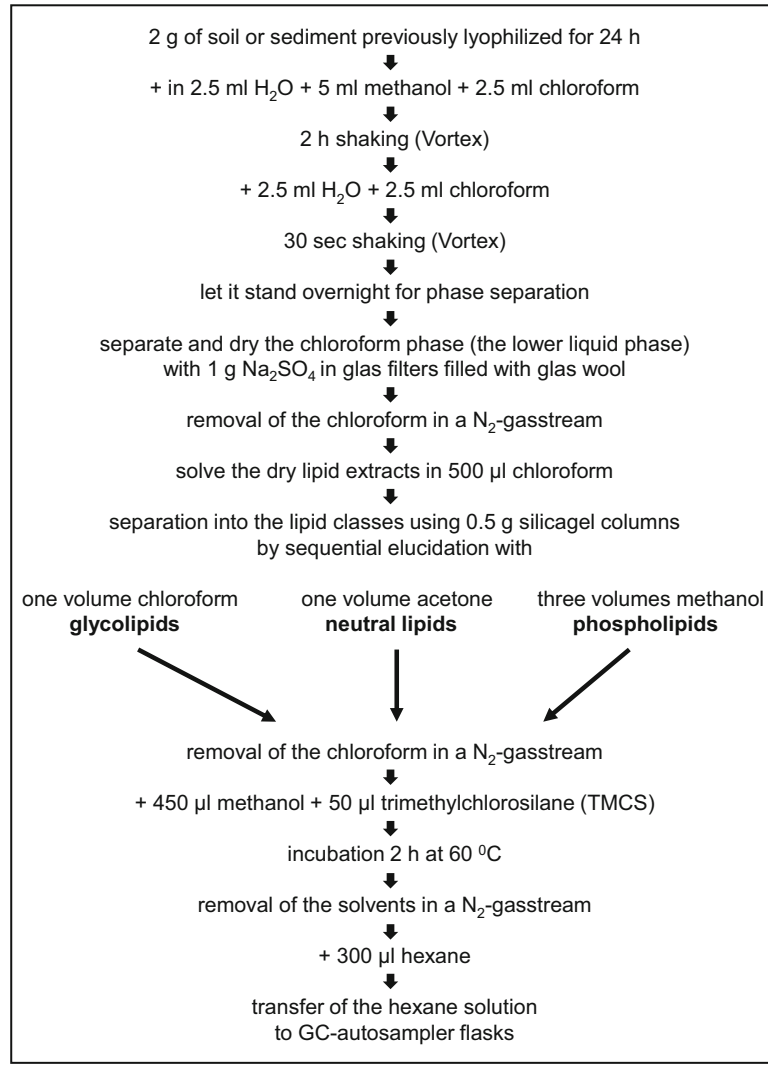

Fig. 1 Scheme of the lipid extraction of soil and sediment samples, their separation in neutral lipids, glycolipids and phospholipids and the conversion of fatty acids to fatty acid methyl esters (FAMEs) as routinely performed for analysis of lipids from environmental samples

3.2 Procedures

3.2.1 Total Lipid Extraction

For the extraction of total lipids, 2 g of lyophilised soil was transferred to clean glass vials. Lyophilisation was previously performed for 24 h to get rid of the remaining water that would disturb the ratios of the solvents in the following extraction. The lipids of the lyophilised soil are extracted subsequently with chloroform/methanol/water (1:1:1 v/v) as first described by Bligh and Dyer [15] and shown schematically in Fig. 1. After phase separation, the lipid-containing chloroform extracts are dried using anhydrous sodium sulphate (Na_2SO_4) which is filled into a layer of glass wool on a

glass filter to remove remaining water. The chloroform is then removed in a N_2 gas stream and the dried extracts dissolved in a small volume of chloroform.

3.2.2 Separation of the Total Lipids into Neutral Lipids, Glycolipids and Phospholipids

Afterwards, the total lipid extract is separated into the different lipid classes by liquid chromatography using silica gel columns (Baker-bond spe, Baker) and subsequential elution using different solvents. Elution with one volume chloroform (5 mL/0.5 g silica) results in the neutral lipid fraction. Elution with one volume acetone (5 mL/0.5 g silica) results in the glycolipid fraction. Elution with three volumes of methanol (15 mL/0.5 g silica) results in the phospholipid fraction. All fractions should be collected separately. The major fraction for characterisation and detailed analysis of microbial communities is the phospholipid fraction, the phospholipid fatty acids (PLFA). However, the further analysis of the two other fractions can be of specific interest, depending on the research questions [9]. The solvents of the lipid fractions of interest are evaporated in a N_2 gas stream.

3.2.3 Methylation to Fatty Acid Methyl Esters (FAMEs)

Next step is the transesterification of the dry fatty acids which are still esterified to glycerol to their corresponding trimethylsilyl esters (TMSE) with 500 µL trimethylchlorosilane (TMCS) in methanol (1:9, v/v) at 60°C for 2 h. The solvent is then evaporated under a stream of N_2 gas, and residues are resuspended in hexane for GC analysis. Figure 1 presents a scheme of the overall procedure.

A second method is the transesterification of dry PLFA extracts with boron trifluoride in methanol complex (BF_3 in MeOH) to their corresponding fatty acid methyl esters (FAMEs) according to the method of Morrison and Smith [16]. Methylation is carried out with 1.2 mL BF_3 in MeOH at 95°C for 15 min. Reaction is stopped by adding 0.6 mL distilled water and 1 mL n-hexane. After shaking for 60 s, the entire upper phase is transferred to a clean glass vial and the solvent is evaporated under a stream of nitrogen. The dried sample is dissolved in 1 mL n-hexane including internal standard (e.g. heneicosanoic acid methyl) of known concentration (e.g. 10 µg/mL) and stored at 5°C until analysis via gas chromatography. The addition of an internal standard is not necessary for the relative quantification of fatty acids but allows the calculation of their absolute amounts. Compared to the boron trifluoride methylation, the treatment with TMCS is the less aggressive method and is therefore applied in order to detect hydroxy fatty acids as well.

3.2.4 Analysis of Fatty Acid Composition by GC-MS and GC-FID

Analysis of TMSE or FAMEs in hexane is performed using a quadrupole GC-MS System (HP8690, Hewlett-Packard, Palo Alto, USA) equipped with a split/splitless injector. In our laboratory, a BPX-5 capillary column (SGE, Darmstadt, Germany; length, 30 m; inner diameter, 0.32 mm; 0.25 µm film) is used for separation where the injector temperature is held at 240°C. The injection is

splitless and He is used as carrier gas at a flow of 2 mL/min. The temperature programme is 40°C, 2 min isothermal; 4°C/min to 230°C; and 5 min isothermal at 230°C. The pressure is held constant at 7.57 psi.

In addition, a GC system with flame ionisation detector (Agilent 6890N) with a special FAME column (CP-Sil88 Varian Chrompack, length, 50 m; inner diameter, 0.25 mm; 0.2 μL film) is used to achieve better separation of the FAME. The pressure programme is as follows: start: 27.64 psi for 2 min, increase: 0.82 psi/min up to 45.7 psi, isobaric for 5.5 min. The temperature programme starts at 40°C (2 min), increases 8°C/min up to 220°C and is held there for 5 min. Injector temperature is 240°C and detector temperature 270°C.

The peak areas of the corresponding fatty acid trimethylsilyl or methyl esters (TMSEs or FAMEs, depending on transesterification reagent) in total ion chromatograms (TIC) are used to determine their relative amounts. The fatty acids are identified by their mass spectra and retention time compared to co-injections of authentic FAME reference compounds obtained from Supelco (Bellefonte, USA).

3.3 Troubleshooting

In the Bligh and Dyer procedure, it is necessary to obtain a clear chloroform phase (the lower liquid phase). In cases of higher biomass content, this phase can be small. This can be solved simply by adding more (about 2 mL) chloroform to the tubes. The amount of solvents applied for the Bligh and Dyer extraction (always in their fixed ratios of 1:1:1) has to be adjusted to the samples received from the research site of interest.

3.4 Data Analysis of the Phospholipid Fatty Acid Profiles Obtained from Environmental Samples

Generally, the above-described protocol can be used to analyse any environmental sample. However, when looking at the literature, a clear tendency of over-interpretation of the obtained fatty acid analysis data with respect to a direct proof of the presence of certain microorganisms can unfortunately be observed. This problem was recently emphasised in a very helpful overview publication [5]. In fact, most bacteria consist of very similar fatty acid profiles. Therefore, only in very few specific environments can a PLFA analysis allow a direct identification and quantification of certain microorganisms with the help of specific marker fatty acids. As changes in lipid profiles can be caused by changes in the overall community as well as by reactions of the already present microorganisms to environmental stressors, it is very difficult to state that modifications of the PLFA profiles in environmental samples are caused by the occurrence of a certain bacterial genus or species. However, PLFA analysis offers a very powerful tool for the overall identification and quantification of community structures [17]. The general rules for such interpretations can be summarised as follows.

Membrane fatty acids are regularly bound to phospholipids. Thereby, their chain length varies between C_{12} and C_{20}. The membranes of all living systems consist of very similar lipid composition that varies only with respect to chain length, position and configuration of double bonds (unsaturation) and the occurrence of branches; the major exceptions are the Archaea that contain ether-linked alkanols instead of fatty acids.

As phospholipids only occur in the membranes of living organisms and are immediately degraded to neutral lipids by phospholipases when the cells die, the overall PLFA abundance indicates the amount of active, living biomass. Total PLFAs can be used to estimate total numbers of cells present in a sample. The conversion factor of $5.9 * 10^4$ cells pmol of $PLFA^{-1}$ is often used, derived from an average value of 10^4 mol of PLFA g (dry weight) of $cells^{-1}$ [18]. The increase in living microbial biomass could be confirmed in soils by comparing the PLFA data with total DNA extract concentrations and colony-forming unit (CFU) measurements, which all point to the same amounts of living microbial biomass [19].

Generally, bacteria only contain monounsaturated fatty acids. Polyunsaturated fatty acids such as linoleic acid (C18:2 Δ9*cis*,Δ12*cis*) and linolenic acid (C18:3 Δ9*cis*,Δ12*cis*,Δ15*cis*) only occur in eukaryotic membranes or in a very few bacteria [20] and are thus a direct biomarker for the presence of eukaryotes in environmental samples. Among the monounsaturated fatty acids, the content of *cis*-vaccenic acid (18:1*cis*Δ11) indicates the presence of a certain group of Gram-negative bacteria such as enterobacteria and pseudomonads containing the so-called anaerobic pathway of fatty acid synthesis [21]. The amount of oleic acid (18:1*cis*Δ9) indicates the presence of a certain group of Gram-negative and all Gram-positive bacteria containing the so-called aerobic pathway of synthesis of unsaturated fatty acids by desaturases [21]. Branched fatty acids only occur in Gram-positive bacteria and sulphate-reducing bacteria (SRB) [7, 22]. An increase in the ratio of branched to monounsaturated PLFA is caused by a shift in the proportions from Gram-positive to Gram-negative bacteria [13, 23, 24]. Cyclopropane fatty acids indicate starvation in Gram-negative bacteria [25]. *Trans*-unsaturated fatty acids occur in bacteria of the genera *Pseudomonas* and *Vibrio*, some methanotrophic bacteria, *Alcanivorax borkumensis* and several *Nitrosomonas* species [26]. Recently, a new monounsaturated 10-methylhexadecanoic acid (10Me16:1Δ7) unique in *Candidatus Methylomirabilis oxyfera*, a bacterium able to use methane for nitrite reduction, was identified [27].

Figure 2 presents a summarising scheme of the main, overall accepted interpretations of fatty acid profiles regarding the presence of certain microorganisms as well as shifts in the bacterial microbiota. Using these criteria, the PLFA profiles of most environmental samples can be interpreted especially regarding changes in the microbiota caused by certain biological treatments or changes in environmental factor such a physical stresses.

Observation	Interpretation
Amount of phospholipid fatty acids (PLFA)	Content of living biomass
Mono-unsaturated fatty acids	Gram-negative bacteria
Content of 18:1*cis*Δ11 (*cis*-vaccenic acid)	Gram-negative bacteria containing the so-called anaerobic pathway of fatty acid synthesis
Content of 18:1*cis*Δ9 acid)	Gram-negative and all Gram- (oleic positive bacteria containing of the so-called aerobic pathway of fatty acid synthesis using desaturases
Branched fatty acids	Gram-positive bacteria and sulphate-reducing bacteria (SRB)
Ratio of branched / mono-unsaturated fatty acids	Shift from Gram-positive to Gram-negative bacteria
Cyclopropane fatty acids	Starvation in Gram-negative bacteria
trans-unsaturated fatty acids	Stress indicator in bacteria of the genera *Pseudomonas* and *Vibrio*
Poly-unsaturated fatty acids (C18:2 Δ9*cis*,Δ12*cis* = linoleic acid C18:3 Δ9*cis*,Δ12*cis*,Δ15*cis* = linolenic acid	Presence of Eukaryotes

Fig. 2 General overview of the interpretation of data obtained from lipid fatty acid profiles with respect to the community structure or community shifts in environmental samples

3.5 Statistical Analysis

For the interpretation of a series of samples, e.g. for the analysis and monitoring of large contaminated sites, a statistical analysis in the form of a principal component analysis (PCA) should be applied. We performed this on the bases of numerical data matrices converted using the program R (R: Copyright 2005, The R Foundation for Statistical Computing Version 2.1.1). The relative amounts of PLFA data are subjected to PCA in order to investigate the interrelationships between the soil samples and to determine the predominant PLFA species in the samples. This can be carried out in order to identify the environmental factor leading to changes in the PLFA patterns as well as to identify the specific PLFA that changes its content in certain samples [19].

4 Research Outlook

The routine analysis of PLFA profiles has become a standard procedure for the analysis and identification of bacteria and bacterial fatty acids in medical isolates using the Sherlock Microbial Identification System (MIDI Inc., Newark, Del.) or GC-MS systems. Here, enormous FAME databases are existing for a quick identification using GC-FID analysis. Unfortunately, this is not yet the case regarding environmental samples and the fatty acids of environmental bacteria, respectively. Therefore, the identification of marker fatty acids that are specific for a certain group of bacteria is still limited to a few very specific bacteria. Therefore, many research efforts and database formation will still be necessary until a routine protocol for a fast analysis of microbiota from environmental samples will be existing. However, one has always to take into account that a direct identification of certain microorganisms is hindered by the fact that changes in lipid profiles can be caused either by changes in the overall community (community adaptation) or by reactions of the already present microorganisms to environmental stressors (cell adaptation). Therefore, it is very difficult to state that modifications of the PLFA profiles in environmental samples are caused by the occurrence of a certain group of bacteria. For interpretation of data obtained by lipid-based stabile isotope probing methods, which is useful for the interpretation of carbon utilisation in natural environments [28], the effect and position of the tracer should be kept in mind which may also influence the lipid composition [29]. Therefore, other methods such as cultivation techniques or molecular biological tools have to be used for a qualitative and quantitative investigation of changes in the microbiota. The combination of PLFA and 16S rRNA analysis allows the study of microbial diversity and their physiological state in one experiment [7]. For an overall analysis of the microbiota in environmental samples, a polyphasic approach in microbial ecological investigations in the form of combinations of culture-dependent (e.g. most probable number, MPN) and several culture-independent methods (16S rRNA-based profiles, DNA arrays, PLFA analysis, clone libraries) will be necessary to get a reasonable quantitative and qualitative overview of microbiota changes in environmental samples such as soils contaminated with hydrocarbons during a bioremediation treatment. The diagnostic potential of phospholipids may also be beneficial for the systematic design of a complete set of biomarkers for various microbial foodborne human pathogens [30].

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Protocols for Purifying and Characterizing Integral Membrane AlkB Enzymes

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Abstract

Alkane-oxidizing bacteria metabolize much of the aliphatic content of petroleum that is released into the environment and therefore play a significant role in the biogeochemical cycling of carbon. The enzymes used by these organisms are of significant interest because of their ability to oxidize inert saturated hydrocarbons. This protocol describes two approaches to express, purify, and characterize active alkane monooxygenase (AlkB), the metalloenzyme that catalyzes the oxidation of most medium-chain-length alkanes in the environment. One method uses *P. putida,* grown on octane, as an expression host to produce AlkB. The second method uses affinity-tagged AlkB recombinantly expressed in *E. coli*. After purification from either source, iron reconstitution is often necessary to achieve maximal activity. AlkB activity is determined by using a simple epoxidation assay or by using radical clock substrates, which can also provide mechanistic detail. Both assays require the use of proteinaceous electron donors, which utilize NADPH or NADH depending on the reductase. The native reductase is easily purified, or commercially available substitutes may be used.

Keywords: Alkane monooxygenase, Alkane oxidizing, Alkanotrophs, AlkB, Hydrocarbonoclastic

1 Introduction

The alkane monooxygenase (AlkB) family of integral membrane diiron hydroxylases is responsible for catalyzing the hydroxylation of medium- to long-chain alkanes, 5 to 22 carbons, in the environment. Functional AlkBs have been isolated from marine, soil, and pathogenic bacterial species. Though the AlkB of *Pseudomonas putida* GPo1 remains the best characterized of these enzymes, more than 450 putative AlkB genes have been identified across seven bacterial phyla (1–7). Recombinant systems have been used to express a few AlkBs, but the approach has not been widely adapted to explore the diversity of the AlkB family.

While AlkB enzymes oxidize terminal alcohols into aldehydes, epoxidize terminal olefins, and demethylate methyl ethers, they

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have attracted particular attention for their ability to hydroxylate terminal carbons of linear and branched alkanes (8). The conversion of chemically inert and environmentally toxic alkanes into biologically accessible oxidation products has numerous potential applications, including the bioremediation of oil spills. Many of the marine bacteria that produce AlkBs, such as *Alcanivorax borkumensis,* already fill this role in nature, proliferating in environments that are contaminated with crude oil (6, 9).

AlkB-containing organisms have also been widely explored for their potential as biocatalysts for industrial processes (10–24). *Pseudomonas putida* GPo1 (formerly known as *Pseudomonas oleovorans*) produces surfactants when grown on alkanes, prompting bioengineering efforts to exploit these complex biomolecules (18–20). Despite the potential advantages of overexpressing AlkB in *E. coli*, problems with recombinant expression have been detected. Fully metallating the dinuclear-iron enzyme in a nonnative host is difficult (24), and problems with cellular localization have been reported (12).

An in-depth understanding of AlkB structure, selectivity, and mechanism has been limited by the challenges associated with expressing and purifying membrane proteins. AlkBs, like many integral membrane proteins, express poorly and are difficult to stabilize for the duration of a purification protocol. Successful purification protocols have been developed for AlkB and fall into one of three categories: detergent-free vesicle-based purification, affinity-tagged purification in the presence or absence of detergent, and detergent-solubilized purification utilizing ion exchange. Functional analyses can be done on whole cells, cell-free extracts, or purified enzymes (25). Successful purifications of AlkB and the associated specific activities of the final preparations are shown in Table 1. Most specific assays are reported in terms of micromoles of NADPH oxidized per minute per mg of protein. Some researchers, wishing to avoid artifacts from NADPH uncoupling, report specific activities in terms of micromoles of product produced over the course of a 5–10 min assay. Unless otherwise specified, Table 1 reports specific activities in terms of NADPH consumption. All of the published procedures, except for our recent publication (26), report purification of AlkB from *P. putida* GPo1, the soil bacterium from which AlkB was first identified.

Since isolation of pure enzyme is essential for various structural and mechanistic analyses, in this chapter we describe two different protocols for isolating active AlkB, a single assay for analyzing the enzyme's function, and a method for quantifying the iron occupancy of the enzyme. The first purification approach involves expression of the tagless protein in *P. putida* and relies on centrifugation and detergent solubilization with ion exchange chromatography and size-exclusion chromatography. The second approach

Table 1
Summary of purification approaches, activity assay conditions, and specific activities as reported in the literature

Purification technique	Concentrations of assay components				Specific activity (μmol NADPH oxidized/min/mg AlkB)	References
	[AlkB] (M)	[Rubredoxin] (M)	[Rubredoxin reductase] (M)	[NADPH] (M)		
Precipitation and chromatography	4.4×10^{-6}	2.4×10^{-6}	4.2×10^{-7}	2×10^{-4}	0.133	(27)
Precipitation and chromatography	4.5×10^{-6}	2.2×10^{-8}	1.5×10^{-6}	5.6×10^{-6}	0.03[a]	(28)
Differential centrifugation and cation exchange chromatography	6.9×10^{-6}	7.3×10^{-6}	1.34×10^{-6}	240×10^{-6}	5.2	(29)
Differential centrifugation and cation exchange chromatography	2.2×10^{-7}	1.1×10^{-6}	2.8×10^{-7}	2.5×10^{-4}	4.3	(30)
Detergent solubilization and anion chromatography	7×10^{-6}	7.3×10^{-6}	1.34×10^{-6}	2.4×10^{-5}	4.5[a]	(26)
Detergent solubilization and affinity chromatography	6×10^{-6}	3×10^{-6}	2.5×10^{-7}	5×10^{-5}	0.04	(31)
Affinity chromatography	2×10^{-6}	3×10^{-6}	6×10^{-7}	2×10^{-4}	2	(32)

[a]Activity is reported in terms of micromoles of product per mg of protein after 10 min reaction. Information about the micromole NADPH oxidized per minute is not available

involves heterologous expression of the tagged construct in *E. coli* followed by affinity chromatography.

Both approaches have been used to produce very pure active protein. Growing *P. putida* on an alkane to induce the expression of AlkB is preferable for applications that can be done in partially purified cell extracts because these cells contain the companion electron transfer proteins (obviating the need for purifying those proteins separately) and consistently yield higher levels of activity without the need for iron reconstitution. Overexpressing tagged constructs in *E. coli* generates purified protein more rapidly than the native system and enables mutagenesis studies. Additionally, considerably higher yields of pure protein are obtained per liter when recombinant *E. coli* strains are grown on EnpressoB than are obtained when *P. putida* is grown on an alkane. The EnpressoB-mediated growth process is also much more rapid, requiring slightly less than 2 weeks to go from a single bacterial colony on a plate to a few mg of highly pure active protein. However, tagged proteins require metal reconstitution to show levels of activity that parallel those shown for native protein purified from cells grown on alkanes.

2 Materials

2.1 Purification of Native AlkB

2.1.1 Cell Culture

1. Growth media: Luria broth (LB), minimum salts basal media (MSB) (33)
2. Appropriate antibiotics
3. Octane, for the carbon source (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)

2.1.2 Purification

1. Tris–HCl buffer with 0.04% (w/v) n -dodecyl-β-D-maltopyranoside (DDM) (20 mM Tris–HCl, pH 7.4, 0.94 mM dithiothreitol (DTT), and 0.38 mM phenylmethylsulfonyl fluoride (PMSF))
2. Detergent: *n*-dodecyl-β-D-maltopyranoside (DDM) (*see* Sect. 2.6)
3. Sonication device: ultrasonic liquid processor
4. Bradford Protein Assay (Bio-Rad http://www.bio-rad.com)
5. 5 ml HiTrap Q FF anion exchange column (GE Healthcare http://www.gelifesciences.com)
6. ÄKTA Purifier UPC10 FPLC (GE Healthcare (http://www.gelifesciences.com))
7. HiLoad 16/600 Superdex 200 pg size-exclusion column (GE Healthcare (http://www.gelifesciences.com)
8. Binding buffer (0.04% (w/v) DDM, 20 mM Tris–HCl, pH 8.0)

9. Elution buffer (0.04% DDM, 20 mM Tris–HCl, pH 8.0, 0.5 M NaCl)
10. 7 ml Wheaton dounce tissue grinder (http://www.wheaton.com)
11. 100 kDa molecular weight cutoff MWCO concentrators

2.2 Purification of Recombinant AlkB Using Strep Tag

2.2.1 Cloning

1. Primers:

   BsaI-Strep-AlkB-For: 5′-GGAGATATACAAATGGCTAGCT GGAGCCACCCGCAGTTCGAAAAAGGC GCC-AlkB sequence-3′
   BsaI-AlkB-For: 5′-GGAGATATACAAATG-AlkB sequence-3′
   BsaI-AlkB-Rev: 5′-GGTGGCTCCAAGCGCTTATTA-AlkB sequence-3′
2. Gel and PCR DNA purification kit: NucleoSpin® Gel and PCR Cleanup Kit (Clontech http://www.clontech.com).
3. Vector: pPR-IBA1, previously linearized with BsaI restriction enzyme (IBA Life Sciences (http://www.iba-lifesciences.com).
4. Gene for the alkane hydroxylase of interest.
5. Cloning kit: In-Fusion® HD Cloning Plus Kit (Clontech http://www.clontech.com).
6. PCR Master Mix: AmpliTaq® Gold 360 Master Mix (Life Technologies http://www.lifetechnologies.com).
7. Bacteria strains: E. cloni® 10G Chemically Competent Cells (Lucigen http://www.lucigen.com) and *E. coli* C41 DE3 strain are used for cloning and expression.
8. Super optimal broth with catabolite repression (SOC medium) (Invitrogen http://www.lifetechnologies.com).
9. QIAgen Spin Miniprep Kit (Qiagen http://www.qiagen.com).

2.2.2 Cell Culture

1. Growth media: EnPresso B (Biosilta http://www.biosilta.com)
2. Antibiotics: ampicillin (*see* Sect. 2.6)
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (*see* Sect. 2.6)
4. Ferric citrate (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/): 0.5 M

2.2.3 Purification

1. Detergent: DDM (*see* Sect. 2.6)
2. 5 ml StrepTrap HP column (GE Healthcare http://www.gelifesciences.com)
3. Binding buffer: 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.007% (w/v) DDM
4. Elution buffer: 100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.007% DDM, 2.5 mM desthiobiotin
5. Superdex™ 200 Increase 10/300GL size-exclusion column (GE Healthcare http://www.gelifesciences.com)

2.3 Quantification of Iron Content

1. Nitric acid HNO_3
2. Chelex® 100 sodium form (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)
3. 10 ml gravity-flow polypropylene columns (Thermo Scientific http://www.thermoscientific.com)
4. Pure, detergent-solubilized alkane hydroxylase
5. Dithionite $Na_2S_2O_4$ (1 M stock)
6. Ferrous ammonium sulfate hexahydrate $(NH_4)_2Fe(SO_4)_2 \cdot 6 H_2O$ (1 M stock)
7. HiTrap 5 ml desalting column (GE Healthcare http://www.gelifesciences.com)

2.4 Probing Mechanistic Function Using Radical Clock Substrates

1. Bicyclo[4.1.0]heptane norcarane (34, 35)
2. Deuterated chloroform stabilized with Ag (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)

2.5 Activity Assay

1. Substrate: Octadiene (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)
2. Products: 1,2-Epoxy-7-octene and 1,2,7,8-diepoxyoctane (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)
3. Activated alumina (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)
4. AlkG (rubredoxin from the alk operon) (36)
5. Reductase (*see* **Note 2**)
6. Deuterated chloroform stabilized with Ag (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)
7. NADPH (60 mM stock) (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/)

2.6 General Buffers and Reagents

1. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/): 100 mM in ethanol. Store at −20°C.
2. Dithiothreitol (DTT) (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/): 1 M. Store at −20°C.
3. Ampicillin antibiotic (Sigma-Aldrich http://www.sigmaaldrich.com/united-states.html/): 100 mg/ml in water. Store at −20°C.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fischer Scientific http://www.fischersci.com): 1 M. Store at −20°C.
5. *n*-Dodecyl-β-D-maltopyranoside (DDM) (Anatrace http://www.anatrace.com). Store at −20°C.

3 Methods

The protocols below describe how to purify AlkB from *P. putida* or *E. coli* expression systems. The procedure for the purification of AlkB from *P. putida* should be broadly applicable to other AlkB-expressing organisms. The procedure that relies on affinity-tagged, recombinant AlkB, expressed in *E. coli*, would likely require optimization for each AlkB being expressed.

3.1 Native Alkane Monooxygenase (AlkB) Expression and Purification

This method has been employed to purify the AlkB of *Pseudomonas putida* GPo1 and *Alcanivorax borkumensis*, both expressed in *P. putida* GPo1. This expression system was developed by Jan B. van Beilen (37) and has been used to express a number of different AlkB homologues (38, 39). Recombinant cell lines are created by deleting the *alkB* gene that resides on the native OCT plasmid from *P. putida* GPo1 and replacing it with genes for other *alkBs* and for antibiotic resistance. For a more complete description of the construction of this expression system, see the van Beilen references cited above. Alternatively, native AlkB from *P. putida* can be expressed simply by growing *P. putida* strains on alkanes. The cells grow optimally at 30°C and are induced by the native *alkB* promoter. Optimal activity is detected when cells are actively growing (cells are harvested at an optical density at 600 nm of 1). Under these conditions, 1 l of culture yields 1 g of cells. One gram of cells ultimately yields slightly less than 1 mg of extremely pure, active protein.

3.1.1 Cell Culture

1. Grow recombinant *P. putida* cells on Luria broth agar plates using the appropriate antibiotic (37). Incubate plates for 12–24 h. (When native organisms are used, they must be grown on minimal media plates with an appropriate alkane source).
2. Transfer a colony to an Erlenmeyer flask containing 50 ml of minimal salts basal media (MSB) and feed the cells with octane using a vapor diffusion apparatus. No extra iron is added to these cultures (*see* **Note 1**).
3. Grow cells to an OD_{600} of 1.0, and then add the contents of the 50 ml flasks to 400 ml of MSB in 2.4 l flasks.
4. Grow these flasks, shaking (250 rpm) at 30°C, adding 1.5 ml of octane daily.
5. At $OD_{600} = 1.0$, harvest cells by centrifugation at 10,800 × *g* for 15 min at 4°C.
6. Store cell pellets at −80°C.

3.1.2 Purification

1. Resuspend cell pellets in 1 ml of Tris–HCl buffer (20 mM Tris–HCl, pH 7.4, 0.94 mM DTT, and 0.38 mM PMSF) per gram of cell pellet.

2. Lyse cell suspensions by freezing and thawing in dry ice and ethanol three times before sonicating on ice three times at 30 W for 1 min, resting the mixture on ice for 1 min between sonication steps.
3. Centrifuge lysed cells at 20,000 × *g* for 30 min at 4°C and collect the supernatant
4. Sonicate and centrifuge the supernatant at 270,000 × *g* for 2 h at 4°C.
5. Suspend the pellet in 5 ml of 20 mM Tris–HCl buffer (pH 7.4) per gram and homogenize in a tight-pass tissue grinder.
6. Centrifuge the homogenized solution at 270,000 × *g* for 3 h at 4°C. Store the supernatant from this centrifugation step for detergent-free purification and the pellet (membrane fraction) for detergent-based purification.
7. Suspend the membrane fraction in 7 ml of 20 mM Tris–HCl buffer (pH 7.4) per gram and homogenize a second time in a tight-pass tissue grinder.
8. Determine protein concentration using a detergent-modified Bradford assay, and solubilize the homogenized membrane fraction by adding 1 mg of DDM per mg of protein. Shake gently for 30 min at 4°C.
9. Centrifuge the detergent-solubilized solution at 270,000 × *g* for 30 min at 4°C to remove large, insoluble fragments.
10. Decant the supernatant and centrifuge it overnight (14–16 h) at 270,000 × *g*, 4°C to remove additional, smaller insoluble fragments. Resulting supernatant is further enriched in AlkB.
11. Inject the AlkB-enriched supernatant onto a 5 ml Q FF column attached to an ÄKTA Purifier UPC10 FPLC. Inject about 150 mg of protein in binding buffer (*see* Sect. 2.1) and elute with either a linear gradient or a step gradient of elution buffer (*see* Sect. 2.6). AlkB elutes at approximately 0.25 M NaCl. Analyze elution fractions by SDS-PAGE and the activity assay described below. Collect and concentrate AlkB-containing elution fractions in 100 kDa molecular weight cutoff (MWCO) concentrators (*see* **Note 5**).
12. Inject concentrated elution fractions onto a calibrated HiLoad 16/600 Superdex 200 pg size-exclusion column. Concentrate elution fractions in 100 kDa MWCO concentrators and store at −80°C.
13. Purified AlkB may be exchanged into different detergents by diluting the DDM and AlkB solution with at least 100x the original volume in 20 mM Tris–HCl, pH 7.4, and 4 CMC of the new detergent. Samples should then be incubated for 15 min with periodic stirring at 4°C before being concentrated and stored at −80°C.

3.2 Heterologous Expression and Purification of Affinity-Tagged Alkane Monooxygenase (AlkB)

This method has been employed to purify the AlkB of *Alcanivorax borkumensis* using Strep-affinity tag technology. An N-terminal Strep tag was added to the AlkB gene and inserted into an ampicillin-resistant vector containing a T7 promoter (pPR-IBA1). Expression in EnpressoB growth media following the manufacturer's instructions routinely yields 2.5 mg of pure protein from 400 ml of cell-containing culture. Despite the high cost of the growth medium, savings are incurred by using considerably less ampicillin and IPTG. Expression in *E. coli* requires iron reconstitution to obtain active protein.

3.2.1 Cloning

This cloning protocol is useful for adding affinity tags to the gene of interest and cloning it into an inducible vector that can be expressed in *E. coli*.

1. Amplify the gene of interest by PCR using the BsaI-Strep-AlkB-For and BsaI-AlkB-Rev primers (*see* Sect. 2.2). The PCR conditions are 95°C for 2 min, 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The final reaction volume is 50 μl.
2. Purify the PCR product using a PCR Cleanup Kit.
3. Ligate the PCR product into the precut pPR-IBA1 using the In-Fusion® HD Cloning Plus Kit.
4. Transform competent *E. coli* cells and plate them on Luria broth (LB) and ampicillin plates to grow overnight.
5. Perform colony PCR in order to identify successful transformants. The PCR conditions are 95°C for 2 min, 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 30 s. The final reaction volume is 20 μl (*see* **Note 3**).
6. Pick transformant colonies based on the results of colony PCR and grow them on LB and ampicillin plates overnight.
7. Purify transformant plasmids using the QIAgen Spin Miniprep Kit and sequence the insert.
8. Transform *E. coli* C41 cells using the purified plasmids and begin cell culture.

3.2.2 Cell Culture

EnPresso B growth systems yield high quantities of recombinant protein products from relatively small volumes of cell culture.

1. Grow cells according to EnPresso instructions, using ampicillin as the antibiotic. Supplement iron by adding 50 μl of 0.5 M ferric citrate to each 50 ml flask immediately after inducing cells with 1 mM IPTG (final concentration).
2. Harvest cells by centrifugation at 10,000 × *g* for 10 min at 4°C.
3. Store cell pellets at −80°C.

3.2.3 Purification

1. Resuspend cell pellets using 1 ml of Tris–HCl buffer (20 mM Tris–HCl, pH 7.4, 0.94 mM DTT, 0.38 mM PMSF) per gram of cell pellet.
2. Lyse cell suspensions by freezing and thawing in dry ice and ethanol three times before sonicating on ice three times at 30 W for 1 min, resting on ice for 1 min between each sonication step.
3. Centrifuge the lysed cells at 14,200 × *g* for 1 h.
4. Collect the supernatant and centrifuge it at 270,000 × *g* for 18 h.
5. Collect the membrane pellet and solubilize the proteins in 7 ml Tris–HCl buffer with detergent (20 mM Tris–HCl, pH 7.42, 0.17% (w/v) DDM) per gram of membrane by using a tight-pass tissue grinder to homogenize the membrane fraction. Shake the resulting solution at 4°C for 30 min.
6. Centrifuge the membrane solution at 270,000 × *g* for 30 min and collect the supernatant. This should contain solubilized protein.
7. Buffer exchange this solution into binding buffer (*see* Sect. 2.2) four times at 5,000 × *g* for 30 min each using 100 kDa MWCO concentrators.
8. Inject the solubilized protein solution onto a 5 ml Strep-affinity column attached to an ÄKTA Purifier UPC10 FPLC. Inject about 150 mg of protein in binding buffer (*see* Sect. 2.2) and elute with a step gradient of elution buffer (*see* Sect. 2.2). Collect and concentrate elution fractions in 100 kDa MWCO concentrators.
9. Inject concentrated elution fractions onto a calibrated Superdex™ 200 Increase 10/300GL size-exclusion column.
10. Evaluate fractions using native PAGE and the activity assay described below. Concentrated fractions should be flash frozen in liquid nitrogen and stored at −80°C.

3.3 Quantification of Iron Content Using ICP-MS

ICP-OES analysis requires very clean, acid-washed glassware to prevent any trace metals from the glassware contaminating the samples.

3.3.1 Acid Washing Glassware

1. Wash and rinse all glassware in deionized water before placing them in a 70% nitric acid bath overnight.
2. Rinse all glassware three times with deionized water, dry them, and seal them with parafilm.

3.3.2 Deionized Water Trace Metal Purification

1. Pack a 10 ml gravity-flow polypropylene column with Chelex 100 sodium form resin to a bed volume of 9 ml.

2. Rinse the column with at least three bed volumes of deionized water before beginning collection of 300–400 ml of purified water. If a larger volume is required, the Chelex resin should be replaced.

3.3.3 Alkane Hydroxylase Metallation

1. Incubate the detergent-solubilized enzyme in 0.1 M $Na_2S_2O_4$ and 0.1 M $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ on ice for 15 min.
2. Inject the solution onto a 5 ml HiTrap desalting column using Chelexed water as the elution buffer.
3. Collect 0.5 ml fractions and dilute them 1:7 in Chelexed water before ICP-OES analysis. Follow manufacturer's directions to determine where protein will elute.

3.3.4 ICP-OES Iron Quantification

1. Prepare standards ranging from 0 to 100 ppb iron and generate a calibration curve.
2. Introduce alkane monooxygenase samples into the ICP-OES.
3. Determine concentration of protein using a calculated extinction coefficient at 280 nm and compare to concentration of iron from ICP.

3.4 Activity Assays Using Octadiene or the Diagnostic Substrate Norcarane

The rate of octadiene epoxidation is commonly used to quantify AlkB hydroxylase activity. A turbidimetric variant of this assay follows the disappearance of NADPH spectrophotometrically at 340 nm, before quantifying the products generated using a GC-MS. In contrast, the diagnostic substrate norcarane is used to investigate the *mechanism* of alkane hydroxylation. All AlkBs tested to date produce similar amounts of the ring-opened product, hydroxymethyl cyclohexene, and the ring-closed product, norcarnol (40).

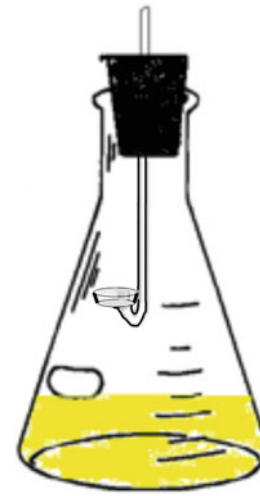
1. If using norcarane, synthesize norcarane and potential products following published procedures (these molecules are not commercially available) (41) (*see* **Note 4**).
2. If using octadiene, purify octadiene before use by passage through an activated alumina column to remove traces of oxidized products that might be present.
3. Into a 1.5 ml microcentrifuge tube, add 320 μg AlkB (final concentration 6.96 μM), rubredoxin (final concentration 7.3 μM), and reductase (final concentration 1.34 μM). Add 10 μl 1 M ferrous ammonium sulfate (final concentration 10 mM) and 10 μl 1 M dithionite (final concentration 10 mM) and incubate on ice for 15 min to ensure full metallation of protein. Bring to a final volume of 1 ml in 20 mM Tris–HCl, pH 7.4, with 0.04% (w/v) DDM.

4. Add 2 μl substrate (final concentration ~20 mM) and 4 μl of freshly prepared aqueous solution of 60 mM NADPH (final concentration 240 μM).
5. Incubate and shake at 37°C for 15 min. The reaction can be followed spectrophotometrically by observing the decay in the NADPH solution at 340 nm.
6. A control experiment should be included, using all reaction components except AlkB.
7. Quench with 250 μl $CDCl_3$ stabilized with Ag.
8. Vortex for 30 s.
9. Centrifuge for 1 min in a table-top centrifuge to separate the aqueous and organic fractions.
10. Inject 1 μl of the organic fraction into GC-MS. Use a HP-5MS cross-linked 5% PH ME Siloxane capillary column (dimensions of 10 m × 0.25 mm × 0.25 μM). Use an injection temperature of 225°C with an initial oven temperature of 50°C and a ramp rate of 10°C per min. Products can be identified by comparison to retention times and fragmentation patterns of authentic standards. Products are quantified by calibration curves made using authentic standards (*see* **Note 6**).
11. Activity is reported in units of micromoles of products per micromoles of protein per unit time.
12. The radical rebound rate can be determined by quantifying the concentration of norcarane-derived ring-opened products, divided by the concentration of norcarane-derived ring-closed products multiplied by the intramolecular rearrangement rate for the norcarane C-2 radical, 2×10^8 s^{-1}. The radical lifetime is the inverse of the enzyme rebound rate. AlkB-catalyzed oxidation of norcarane yields radical lifetimes of 1 ns or greater (40).

4 Notes

1. We fabricated a device for delivering volatile substrates like octane to growing cultures. We used a rubber stopper fitted to the top of the Erlenmeyer flasks used to culture cells that had a glass bulb suspended from it with an open top. The glass bulb, when partially filled with octane, allows the octane to partition into the gas phase and then slowly diffuse into the growth medium. This method of delivering octane to the cells maintains a steady concentration of octane in the growth media during cell growth. Octane levels never reached levels that were toxic to any of the organisms we tested.

2. Our protocol uses maize ferredoxin, which we purified following a published procedure (42). Spinach ferredoxin has also been shown to function in electron transfer to AlkB from *P. putida* (43) and is available from Sigma-Aldrich.



3. To do colony PCR, pick a single colony with a sterile pipette tip. Streak a plate containing the appropriate antibiotic(s) and then allow the pipette tip to rest in 50 μl PCR grade water for approximately 10 min. Use 6 μl of this solution for PCR.
4. The mass spectral characterization data for the AlkB-catalyzed products of the substrates norcarane and octadiene, both used to characterize the reactivity of AlkB, are provided here. For a qualitative assessment of reactivity, this characterization data might be sufficient as product synthesis is time-consuming and not well suited for a lab without synthetic chemistry expertise.
5. DDM micelles are approximately 85 kDa, so the weight of the AlkB monomer plus DDM micelle is larger than 100 kDa. Using a 100 kDa molecular weight cutoff concentrator to concentrate AlkB purified with DDM avoids concentrating empty DDM micelles, which could interfere with downstream uses of the purified AlkB.
6. Characteristic GC-MS fragmentation patterns of norcarane and norcarane-derived products and octadiene products are provided here to assist in identifying these compounds by GC-MS.
    1. Norcarane (34, 35) GC-MS *m/z* (% relative intensity): 96 (22), 81 (98), 68 (48), 67 (100), 55 (41), 54 (55)
    2. Cyclohex-2-enyl methanol GC-MS *m/z* (% relative intensity): 94(38), 53(18), 67(11), 79(72), 81(100)

3. *Endo*-2-norcaranol (bicyclo[4.1.0]heptan-2-ol) GC-MS *m/z* (% relative intensity): 111(12), 55(80), 68(56), 70(63) 77(25), 79(100), 84(91), 97(56)
4. *Exo*-2-norcaranol (bicyclo[4.1.0]heptan-2-ol) GC-MS *m/z* (% relative intensity): 111(4), 57(49), 68(40), 79(100), 84(26)
5. 2-Norcaranone (bicyclo[4.10]heptan-2-one) GC-MS *m/z* (% relative intensity): 54(100), 68(46), 81(47), 95(12) 110(79)
6. 3-Norcaranol (bicyclo[4.1.0]heptan-3-ol) GC-MS *m/z* (% relative intensity): 112(6), 57(13), 67(85), 68(75), 79(100), 94(40)
7. 3-Norcaranone (bicyclo[4.1.0]heptan-3-one) GC-MS *m/z* (% relative intensity): 110(20),54(15), 67(36), 82(28)
8. 3-Cycloheptene-1-ol GC-MS *m/z* (% relative intensity): 57(52), 79(89), 94(78), 95(6)
9. 2-(Hex-5-en-1-yl)oxirane GC-MS *m/z* (% relative intensity): 67(100), 54(91), 55(71), 68(48), 79(38), 93(35), 81(31)
10. 1,4-Di(oxiran-2-yl)butane GC-MS *m/z* (% relative intensity): 57(100), 71(76), 85(57), 99(15), 55(14), 113(9)

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Protocols for Structural and Functional Analysis of Particulate Methane Monooxygenase from *Methylocystis* Species Strain Rockwell (ATCC 49242)

Sarah Sirajuddin and Amy C. Rosenzweig

Abstract

Particulate methane monooxygenase (pMMO) is a membrane-bound metalloenzyme that oxidizes methane to methanol in methanotrophic bacteria, organisms that consume methane as their sole carbon and energy source. In order to understand the biochemistry of methane oxidation, pMMO must be isolated from the native organism. This chapter describes protocols for growth, isolation, and characterization of pMMO from *Methylocystis* species strain Rockwell.

Keywords: Membrane enzyme, Metalloprotein, Methane monooxygenase, Methanotroph, X-ray crystallography

1 Introduction

Methanotrophs are bacteria that use methane as their sole carbon and energy source. The first step in their metabolism, the oxidation of methane to methanol, is carried out by either the particulate or soluble methane monooxygenase (pMMO or sMMO) [1]. Methanol is oxidized to formaldehyde and then to formate by methanol and formaldehyde dehydrogenases followed by either assimilation into cell material or oxidation to carbon dioxide via formate dehydrogenase (Fig. 1) [2]. Because of their unique metabolism, methanotrophs are useful for bioremediation, and understanding the molecular details of methane oxidation by pMMO may inspire technologies enabling the conversion of natural gas into useful chemical feedstocks [3, 4]. This protocol describes a pMMO isolation and characterization procedure for the methanotroph *Methylocystis* species strain Rockwell (ATCC 49242). The *Methylocystis* sp. str. Rockwell genome has been sequenced, and some initial studies on whole-cell metabolism have been reported [5, 6]. Methods for cell growth, pMMO purification, pMMO crystallization, and

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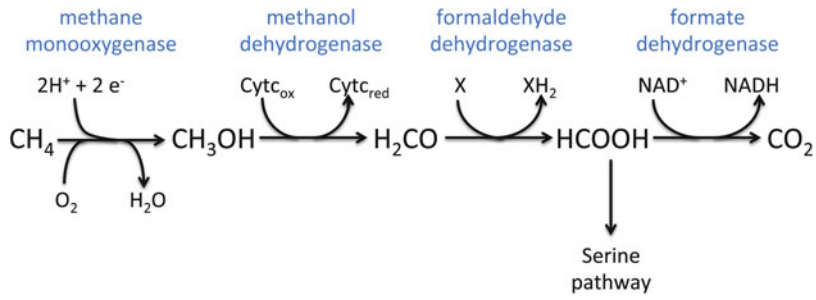

Fig. 1 Metabolic pathway of type II methanotrophic bacteria

enzyme activity are described here and are similar to the isolation and crystallization procedures for *Methylosinus trichosporium* OB3b pMMO [7]. Procedures for isolation of pMMO from *Methylococcus capsulatus* (Bath) have been published previously [8].

1.1 Introduction to Methanotroph Growth

Although pMMO is present in most methanotrophic species, some express both pMMO and sMMO. In these species, sMMO expression occurs at copper levels below 1 μM and pMMO expression is predominant at copper levels above 5 μM [9–11]. This change in gene expression is known as the "copper switch." The mechanism of the copper switch is still unknown, but recent work suggests that the copper chelating natural product methanobactin, genes in the methanobactin operon, and mmoD, a subunit in the sMMO operon, could play a role [12, 13]. Although more experiments need to be carried out to determine whether any of these proteins are indeed involved, the copper switch is likely related to cofactor requirements of the MMO enzymes. sMMO catalyzes the oxidation of methane using a diiron center housed in the hydroxylase protein (MMOH), which contains three polypeptides arranged as an $\alpha_2\beta_2\gamma_2$ dimer. sMMO also requires a reductase protein (MMOR) and a regulatory protein (MMOB) for activity [14]. By contrast, pMMO oxidizes methane using a dicopper center housed in the soluble domains of the pmoB subunit [15, 16]. The structure of pMMO consists of three polypeptides (pmoB, pmoA, and pmoC) arranged as an $\alpha_3\beta_3\gamma_3$ trimer [17]. The genome of *Methylocystis* sp. str. Rockwell contains two copies of the *pmoCAB* operon that encodes pMMO and does not contain the sMMO genes [5]. Although this species does not express sMMO, it should be cultivated in high levels of copper to ensure high pMMO expression and full metal loading of the isolated enzyme. Growth at copper concentrations below 50 μM will likely result in poor metal loading and reduced enzyme activity [10, 18].

1.2 Introduction to pMMO Protein Purification and Crystallization

pMMO accounts for ~80% of the total protein in the inner membranes of methanotrophic bacteria when grown using at least 30 μM copper [19]. Thus, isolation from the native organism is feasible. *Methylocystis* sp. str. Rockwell membrane-bound pMMO is

isolated with 4.2 ± 2 copper ions and 0.4 ± 0.2 iron ions per 100 kDa αβγ protomer [20]. Solubilization of pMMO is required for further purification, but previous studies on *M. capsulatus* (Bath) pMMO show that pMMO activity is inhibited by detergents. The best activity is maintained using n-dodecyl-β-D-maltopyranoside (DDM) at a concentration of 1–1.5 mg detergent/mg protein. Complete inhibition occurs at higher detergent:protein ratios [10, 21]. Although DDM is the best detergent for preserving activity of pMMO, crystallization of pMMO usually requires exchange into a different detergent. pMMO has been crystallized from three species in addition to *Methylocystis* sp. str. Rockwell: *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium* OB3b, and *Methylocystis* species strain M [7, 17, 22]. The *M. capsulatus* (Bath) pMMO was crystallized in the presence of 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5), whereas the *M. trichosporium* OB3b and *Methylocystis* sp. str. M pMMOs were crystallized in the presence of undecyl-β-D-maltopyranoside (UDM). Although different detergents were used, the pMMOs exhibit the same overall architecture.

1.3 Introduction to pMMO Enzyme Activity

pMMO oxidizes only C1–C5 alkanes to secondary alcohols or C2–C4 alkenes to epoxides [21, 23–25]. Activity analysis of pMMO has traditionally been performed using a propylene epoxidation assay, but we now use a methane oxidation assay since methane is the native substrate for growth [8]. Detection of methanol is performed using gas chromatography, and pMMO must be reduced in order to react with substrates. Although the native reductant of pMMO is still in question, it is believed that pMMO receives electrons from the quinone pool. The best reductant for membrane-bound pMMO is NADH, which is thought to provide electrons via a type 2 NADH:quinone oxidoreductase that may feed reduced quinones directly to pMMO [10, 26, 27]. Duroquinol is also a good reductant for membrane-bound pMMO and is the only reductant that is effective after detergent solubilization [21, 28].

Membrane-bound pMMO has higher activity and is more stable than detergent solubilized and purified pMMO. pMMO specific activity is also highest when assayed at low protein concentrations [29]. *Methylocystis* sp. str. Rockwell membrane-bound pMMO exhibits activities typical for a type II methanotroph pMMO when using both duroquinol (4.5 ± 0.9 nmol methanol/min · mg) and NADH reductants (20 ± 3 nmol methanol/min · mg) [7, 20, 22, 30]. After solubilization in DDM, *Methylocystis* sp. str. Rockwell pMMO has an activity of 0.5 ± 0.1 nmol methanol/min · mg. Purified *Methylocystis* sp. str. Rockwell pMMO loses a significant amount of copper upon concentration resulting in an inactive enzyme. It has not been possible to alleviate this metal loss by concentrating anaerobically or by using nonchelating buffers.

2 Materials

2.1 Growth of *Methylocystis* sp. str. Rockwell

1× salt solution (0.085% w/v $NaNO_3$, 0.017% w/v K_2SO_4, 0.0037% w/v $MgSO_4 \cdot 7H_2O$, and 0.0007% w/v $CaCl_2 \cdot 2H_2O$) 390 mM phosphate buffer pH 7.0

0.1 M $CuSO_4 \cdot 5H_2O$

40 mM $FeSO_4 \cdot 7H_2O$ (dissolved in 6.25 mM trace metal grade H_2SO_4)

500× trace elements solution (Table 1)

1 M NaOH

1 M trace metal grade H_2SO_4

25 mM PIPES pH 7.0

Methylocystis sp. str. Rockwell (ATCC 49242) bacterial strain

2.2 Isolation of pMMO-Containing Membranes

Lysis buffer (25 mM PIPES pH 7, 250 mM NaCl)

1 M $CuSO_4 \cdot 5H_2O$

2.3 Purification of pMMO

Purification buffer A (50 mM Tris pH 8.5, 0.05% DDM (Anatrace))

Purification buffer B (50 mM Tris pH 8.5, 2 M NaCl, 0.05% DDM (Anatrace))

100 mg/mL DDM (Anatrace) dissolved in 50 mM Tris pH 8.5

Source 15Q resin (GE Life Sciences)

2.4 Methane Oxidation Activity Assay

Duroquinone

Acidic ethanol (100% ethanol, 3.6 mM HCl)

Sodium dithionite

Table 1
***Methylocystis* sp. str. Rockwell 500× trace elements solution recipe**

500× trace elements	
$ZnSO_4 \cdot 7H_2O$	0.144 g
$MnCl_2 \cdot 4H_2O$	0.083 g
H_3BO_3	0.031 g
$Na_2MoO_4 \cdot 2H_2O$	0.024 g
$CoCl_2 \cdot 6H_2O$	0.024 g
KI	0.0415 g
$CuSO_4 \cdot 5H_2O$	0.0625 g
Doubly distilled water	500 mL

Sodium borohydride

200 mg/mL NADH (Sigma, make fresh before use)

Methanol (Sigma, spectrophotometric grade)

HP-PLOT Q column (Agilent)

Guard column (Restek or Agilent)

2.5 Crystallization of pMMO from Methylocystis sp. str. Rockwell

Crystallization buffer (50 mM Tris pH 8.5, 0.03% UDM (Anatrace))

50% PEG 3000

1 M sodium cacodylate trihydrate

1 M magnesium formate dihydrate

3 Methods

3.1 Growth of Methylocystis sp. str. Rockwell

Cells are grown in 1× salt solution (0.085% w/v $NaNO_3$, 0.017% w/v K_2SO_4, 0.0037% w/v $MgSO_4 \cdot 7H_2O$, and 0.0007% w/v $CaCl_2 \cdot 2H_2O$) with 3.9 mM phosphate buffer pH 7.0, 50 μM $CuSO_4 \cdot 5H_2O$, 40 μM $FeSO_4 \cdot 7H_2O$, and 1× trace metals solution. All solutions should be sterile filtered prior to use, and the salt solution should also be autoclaved before growth. Temperature is maintained at 30°C. Medical grade air and methane gas grade 2.0 (>99% pure) should be used to feed cells.

Methylocystis sp. str. Rockwell is grown in 12–15 L fermentations in a New Brunswick Bioflo 4500 fermenter and should be scaled up from smaller cultures (*see* **Note 1**). Usually 1–4 50 mL cultures are grown in septum-sealed 250 mL flasks (Fig. 2), and headspace gas is displaced with air and methane at a 3:1 ratio once daily (*see* **Note 2**). If seeding culture from frozen cells, resuspend them in 1× salt solution and inject them into the flask using a sterile syringe. An appropriate starting OD_{600} is around 0.05. After cells have fully grown (48 h, OD_{600} ~ 1.0), they are transferred to a 1 L fermenter where air and methane are bubbled in a 3:1 ratio at a total flow rate of 0.16 L/min with 300 rpm agitation. In the late logarithmic phase (OD_{600} ~ 3), the fermentation is pumped into the large 15 L fermenter containing 12 L sterile media. Air and methane are bubbled through this large fermenter at a ratio of 3:1 to a final flow rate of 1.2 L/min, and agitation is kept at 300 rpm. The pH is maintained at 7.0 using 1 M NaOH and 1 M H_2SO_4. Growth can take 2–5 days in the large fermenter. Cells are harvested when they reach an OD_{600} of 5.0–7.0 by centrifugation at 7,000×*g* for 10 min (*see* **Note 3**). Cell pellets are washed by resuspending in 25 mM PIPES pH 7.0 and then spinning down at 7,000×*g* for 1 h three times before freezing in liquid nitrogen and storing at −80°C.

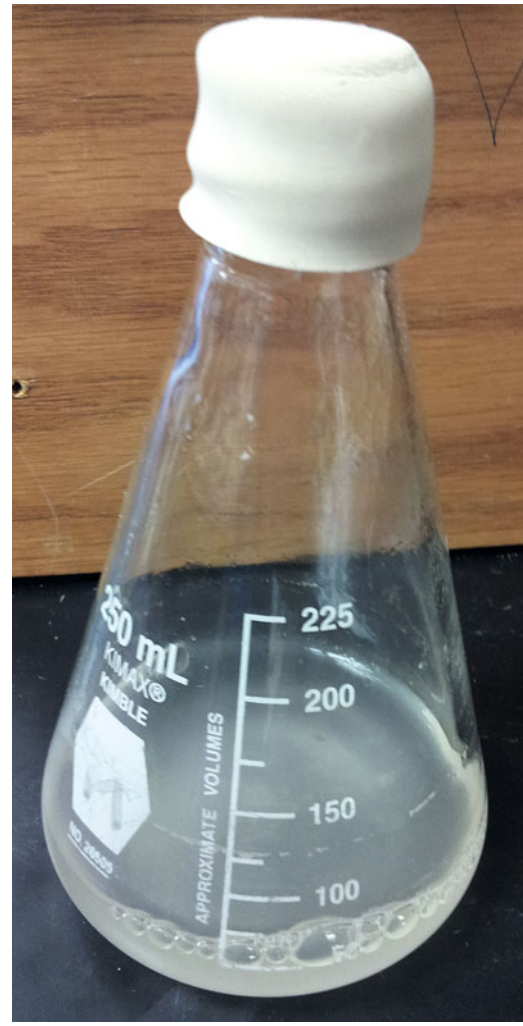

Fig. 2 Septum-sealed flask used for growth of methanotrophic bacteria in a methane/air environment

3.2 Isolation of pMMO-Containing Membranes

1. Lysis buffer (25 mM PIPES pH 7.0, 250 mM NaCl) is degassed under vacuum and argon backfilled. Then 30–50 g *Methylocystis* sp. str. Rockwell cells are thawed in a 125 mL stainless steel beaker with ~100 mL lysis buffer. Once cells are thawed, 500 μM $CuSO_4 \cdot 5H_2O$ is added, and the cells are chilled on ice for 10–15 min.
2. Cells are sonicated for 9 min with a 1 s on and 1 s off time using 40% power.
3. Cell lysate is centrifuged at 24,000×*g* for 2 h at 4°C to remove cell debris.
4. Supernatant is carefully removed and centrifuged at 125,000×*g* for 1 h at 4°C to pellet inner membranes that contain pMMO.

Fig. 3 Example of purification buffer setup. Balloons filled with argon are secured to a bottle containing degassed buffer using a syringe and parafilm. Argon slowly bubbles through the solution via tubing that attaches to the syringe and runs to the bottom of the bottle

5. Membranes are washed two times with 25 mM PIPES pH 7, 250 mM NaCl by resuspending with a Dounce homogenizer and then spinning down in the ultracentrifuge (125,000×*g*, 45 min, 4°C). After washing, the membrane pellet is resuspended to 3–4 mL and frozen in liquid nitrogen.

3.3 Purification of pMMO

1. Purification buffers A (50 mM Tris pH 8.5, 0.05% DDM) and B (50 mM Tris pH 8.5, 2 M NaCl, 0.05% DDM) are degassed for 2 h and backfilled with argon.
2. Crude *Methylocystis* sp. str. Rockwell membranes are thawed and the protein concentration measured by the Bio-Rad DC protein assay (*see* **Note 4**). Membranes are solubilized with 1.5 mg DDM per mg protein by stirring at 4°C for at least 15 min.
3. Solubilized membranes are diluted fourfold in buffer A and purified on a Source 15Q column (GE Life Sciences). Purification is carried out using an ÄKTA FPLC system, and buffers are kept anaerobic by using argon balloons over them (Fig. 3). pMMO usually elutes in multiple peaks, but the protein that will crystallize most reproducibly elutes in the first 1–2 fractions between 0.1 and 0.16 M NaCl. These fractions are

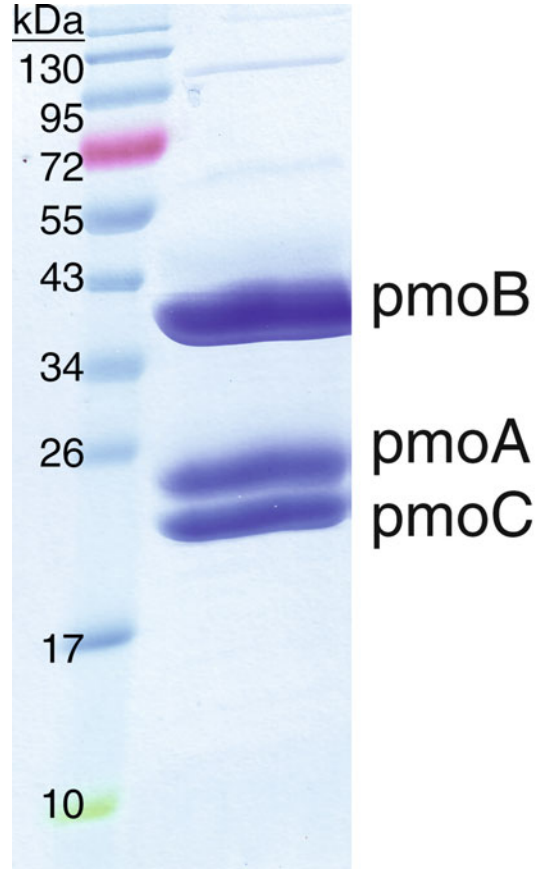

Fig. 4 SDS-PAGE gel of purified *Methylocystis* sp. str. Rockwell pMMO showing the three pMMO subunits (pmoB, pmoA, and pmoC)

concentrated using a 50-kDa-molecular-weight cutoff Amicon concentrator. Purified protein (Fig. 4) can be frozen or used immediately for crystallization.

3.4 Methane Oxidation Activity Assay

This method has already been published for pMMO from *M. capsulatus* (Bath) [8], and the same method is used for *Methylocystis* sp. str. Rockwell. Membrane-bound pMMO can be reduced with either NADH or duroquinol and then reacted with methane. Methanol formation is monitored using an Agilent 7890 Gas Chromatograph and an HP-PLOT Q column. With a 55–250°C ramp at 50°C/min, methanol elutes at approximately 1.6 min. Activity is calculated by comparison with a methanol standard curve (0.0625–1 mM methanol).

1. Duroquinol is prepared by the method of Zahn and Dispirito [18] by dissolving 0.2 g duroquinone in 20 mL acidic ethanol.

Sodium dithionite (0.28 g) is mixed in for 30 min followed by 0.07 g sodium borohydride. Once the solution turns white, duroquinol is precipitated with 200–400 mL water, filtered, and dried for at least 1 h under vacuum. Dried duroquinol can be stored at 4°C for a month.

2. GC reactions are carried out in 2 mL septum-sealed vials. Either 100 μL of crude membranes (5 mg/mL) are mixed with one small scoop of solid duroquinol (1 M final concentration, *see* **Note 5**) or 98 μL of crude membranes (5 mg/mL) are mixed with 2 μL 200 mg/mL NADH (5.5 mM final concentration).
3. 1 mL of headspace gas is removed and replaced with 1 mL of methane gas, and samples are incubated in a shaking water bath at 30°C for 3 min at 200 rpm.
4. Reactions are then placed in an 80°C water bath for 10 min to heat deactivate the enzyme followed by cooling on ice for 20 min.
5. Reactions are transferred to 1.5 mL microcentrifuge tubes and centrifuged for 10 min at max speed at 4°C.
6. Supernatant is pipetted into 250 μL inserts and placed in 2 mL vials with septum-sealed caps. 3 μL of supernatant are injected onto the GC for methanol quantitation using a splitless injection.

3.5 Crystallization of pMMO from Methylocystis sp. str. Rockwell

1. Detergent exchange solution (50 mM Tris pH 8.5, 0.03% UDM) is degassed for 1 h and backfilled with argon gas.
2. Purified pMMO is thawed on ice and detergent exchanged using a 0.5 mL 100-kDa-molecular-weight cutoff Amicon concentrator by diluting with 50 mM Tris pH 8.5, 0.03% UDM, and concentrating three times.
3. The absorbance at 280 nm is measured, and the protein concentration is determined using the calculated extinction coefficient 253,511 M^{-1} cm^{-1} and a molecular weight of 93,000 g/mol. pMMO is then diluted to 20 mg/mL for crystallization.
4. Crystallization buffer is prepared fresh to final concentrations of 10% PEG 3000, 100 mM sodium cacodylate trihydrate pH 6.5, and 200 mM magnesium formate dihydrate.
5. Crystal trays are set up with the sitting tray geometry using 500 μL crystallization buffer in the wells and 1 μL of pMMO plus 1 μL crystallization solution in the drop. pMMO crystallizes in 1–2 weeks at room temperature (Fig. 5).

Fig. 5 *Methylocystis* sp. str. Rockwell pMMO crystals. The longest dimension is 1 mm

4 Notes

1. If ~10 g frozen cells are available, growth can be started at the 12 L volume. Frozen *Methylocystis* sp. str. Rockwell cells are thawed in 50 mL 1× salt solution and added to the 12 L fermenter with a sterile syringe. Using this method, cells are not likely to grow unless the starting OD_{600} is between 0.2 and 0.4.
2. It is wise to have a separate set of flasks for use with methanotrophs that are not used for growth of other organisms or they may grow poorly or not at all.
3. To maximize yield from one growth, ~2 L of cells should be kept in the fermenter after harvest. A new growth can be started from this remaining culture by adding 12 L sterile 1× salt solution media, 3.9 mM phosphate buffer pH 7.0, 50 μM $CuSO_4 \cdot 5H_2O$, 40 μM $FeSO_4 \cdot 7H_2O$, and 1× trace metal solution to the fermenter. This procedure should only be repeated two times after which the next growth should be started from a fresh culture.
4. A 10 μL aliquot of crude membranes are solubilized in 2% (w/v) DDM and then diluted 15- to 125-fold to take the protein concentration.

5. Duroquinol is insoluble in water. By adding a small scoop of solid duroquinol to the reaction vial, the saturation limit is reached and the solution will appear cloudy.

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Phenotype-Based Identification of Key Enzymes for Polycyclic Aromatic Hydrocarbon (PAH) Metabolism from Mycobacteria Using Transposon Mutagenesis and a PAH Spray Plate

Seong-Jae Kim, Ohgew Kweon, and Carl E. Cerniglia

Abstract

Despite considerable knowledge of bacterial aromatic hydrocarbon metabolism, the key enzymes and their pleiotropic and epistatic behaviors responsible for mycobacterial high-molecular-weight (HMW) polycyclic aromatic hydrocarbon (PAH) metabolism remain poorly understood. In this chapter, we describe a rapid, low-cost, high-throughput screening for function-dependent selection of mutants with metabolic discrepancies or defects in the metabolism of HMW-PAHs, which consists of (1) preparation of electrocompetent mycobacterial cells; (2) construction of a transposition mutant library, using a Tn5-based transposon; (3) phenotypic screening, using a PAH spray plate method; and (4) rescue cloning of transposed genomic DNA. The protocol may also be applicable to other PAH-degrading mycobacterial strains or actinobacteria.

Keywords: *Mycobacterium*, PAH metabolism, PAH spray plate, Phenotype, Transposon mutagenesis

1 Introduction

As a class of organic compounds, polycyclic aromatic hydrocarbons (PAHs) are characterized by two or more fused benzene rings arranged in various configurations that do not typically carry other functional groups or branched groups [1]. PAHs are ubiquitous contaminants, being produced petrogenically, pyrogenically, and biogenically, and pose a significant risk to humans and the environment [1]. Microbial degradation is the major mechanism responsible for the ecological recovery of PAH-contaminated sites [1]. The biodegradation of low-molecular-weight (LMW) PAHs with two or three rings by microorganisms has been the subject of many excellent researches [1, 2]. In the last decade, research on the bacterial biodegradation of HMW-PAHs with highly toxic and recalcitrant properties has advanced significantly [3].

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From a historical perspective, the members of the genus *Mycobacterium* have been reported as dominant bacteria, able to degrade HMW-PAHs, and a promising bioremediation tool for ecological recovery [3]. The first bacterium reported to degrade HMW-PAHs, such as four-ringed pyrene and five-ringed benzo[*a*]pyrene, was *M. vanbaalenii* [4–8]. Since its isolation in 1986, other strains of *Mycobacterium* capable of degrading HMW-PAHs have been isolated from PAH-contaminated environments from geographically diverse locations [3]. Enrichment culture using HMW-PAHs as sole sources of carbon and energy has often led to the isolation of *Mycobacterium* spp. The physiology and enzyme systems of mycobacteria are ecologically advantageous, allowing them to survive and degrade HMW-PAHs under various conditions in contaminated soils [4, 9].

Despite its relatively short research history, considerable knowledge of mycobacterial HMW-PAH metabolism has been organized into the PAH metabolic network [4–6, 10–14]. From the initial chemistry studies focused on identification of PAH metabolites, mycobacterial degradation pathways for many PAH substrates have been elucidated [7, 15–24]. Subsequent molecular genetic studies identified and annotated the PAH-catabolic genes and enzymes for the metabolite-centric pathways [4, 9, 11, 14, 24–28]. During the last decade, 66 complete mycobacterial genome sequences have become publicly available (http://img.jgi.doe.gov). Functional genomic studies, such as proteomics, have contributed significantly to our understanding as to how these bacteria degrade PAHs and adapt to the environment [5, 6, 12, 14, 28–34]. Recently, new phenotypic high-throughput screening, by combination of Tn5-based transposon mutagenesis and a PAH spray plate method, has been introduced to selectively screen loss-of-function mutants toward certain PAH substrates [14, 35]. Using mutants able to degrade 4-ring PAHs but not 3-ring and vice versa, the pleiotropic and epistatic functional complexity of ring-hydroxylating oxygenases (RHOs), some of the key enzymes in PAH metabolism, have been uncovered [14, 35]. These studies further suggest that the pleiotropic activity and epistatic interaction (the functional combination of two or more enzymes for a single metabolic step) of the enzymes involved in PAH metabolism should generally be considered for both experimental designs and practical bioremediation applications.

A phenotype-based (or forward genetics) strategy, coupled with a PAH spray plate method, provides a unique way to selectively screen mutants for subsequent study of the pleiotropic and epistatic

behavior of enzymes involved in bacterial PAH metabolism. Tn-5 transposons are a powerful genetic tool to create mutant libraries in a wide range of genera [36, 37]. Such a mutant library allows high-throughput screening aimed at the identification of genes (or enzymes) responsible for defined phenotypes [14, 35]. A spray plate method using PAH substrates is a simple, time-saving, and efficient screen for functional discrepancy or defectiveness of enzymes involved in PAH metabolism [38]. However, there are biological and technical obstacles for construction of a mutant library using Tn5-transposon and for functional visualization of the mutant library using a spray plate method with PAH substrates. Members of the genus *Mycobacterium* are refractory to genetic manipulation due to the presence of a thick and waxy cell wall. Certain genes (or enzymes) may have no obvious loss-of-function phenotype due to: (1) functional redundancy by epistatic contributions from paralogous and other genes, (2) existence of alternative metabolic pathways, and (3) lethality. To overcome these constraints, we conducted repeated trial-and-error studies with changing experiments factors until we had a satisfactory level of transformation efficiency enough to generate thousands of mutants. We used high field strength (2.4 kV) per millimeter cuvette gap and resistance of 200 Ω, which is much stronger than that often recommended for clinical strains of mycobacteria [14]. For selection of a specific mutant which is able to degrade four-ring but not three-ring PAHs, we played two rounds of PAH spray plate tests (first test of the four-ring PAHs fluoranthene and pyrene and second test of the three-ring PAHs phenanthrene, fluorene, and anthracene) on over 4,000 transposon mutants [14, 35]. The successful experimental conditions and screening strategies developed in this study may be helpful as a reference method for PAH metabolism studies on other environmental mycobacteria or other nocardioform *Actinobacteria*.

In this chapter, using a fast-growing, PAH-degrading mycobacterium, we describe a protocol for function-dependent selection of mutants with metabolic discrepancies or defects in HMW-PAH metabolism, including Tn5-based transposition mutagenesis for construction of a mutant library, which is followed by phenotypic screening of mutants using a combination of PAH spray plate method (Fig. 1). The protocol can be used as a reference method for other environmental mycobacteria degrading PAHs or other species in the *Actinobacteria*.

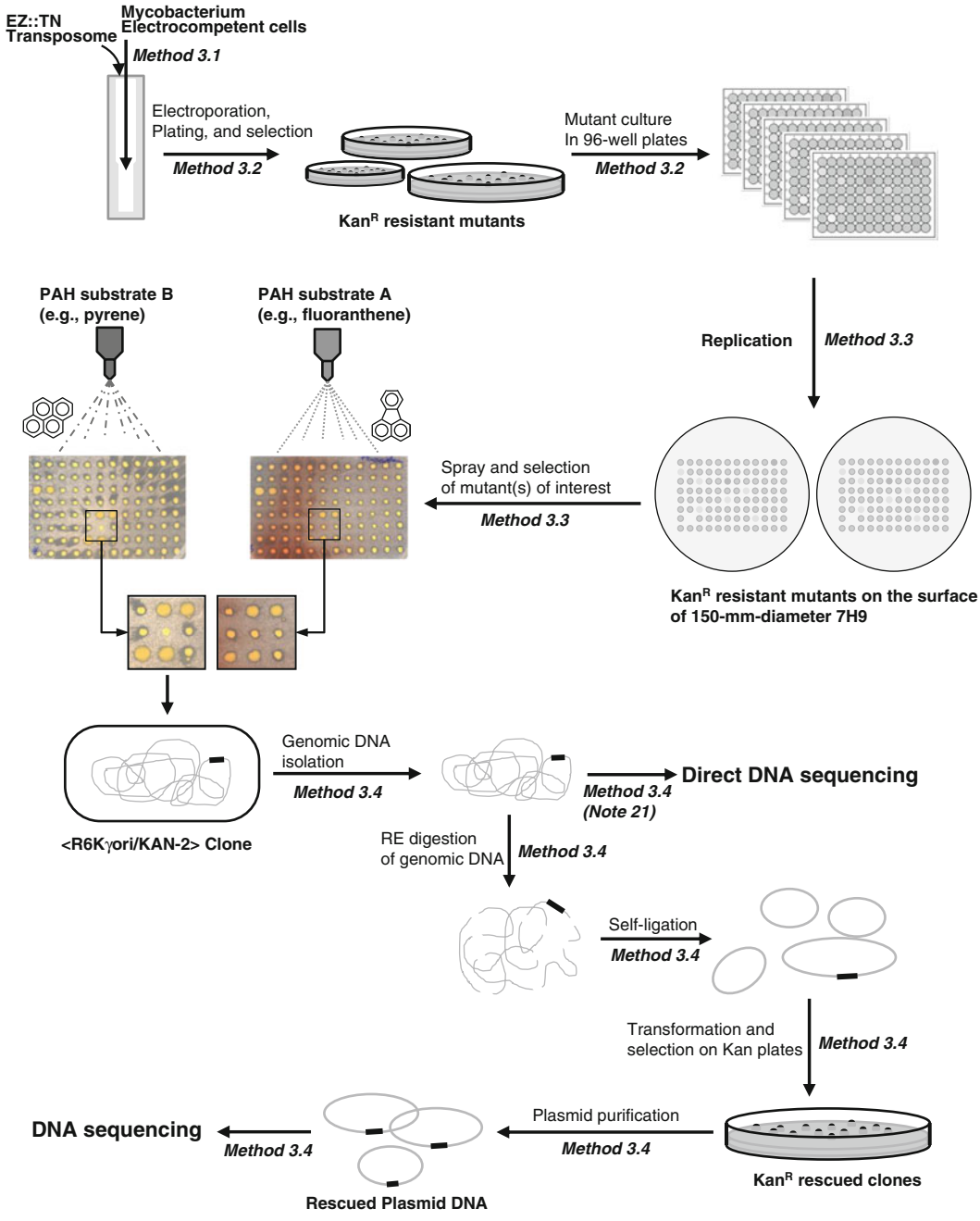

Fig. 1 Function-dependent selection of mycobacterial mutant(s) with metabolic discrepancy or defectiveness to PAH metabolism using Tn-5 mutagenesis and a PAH spray plate

2 Materials

2.1 Preparation of Electrocompetent Mycobacterial Cells

1. Middlebrook 7H10 agar plates with OADC enrichment (Remel, Lenexa, KS).
2. Mycobacterial strain of interest (*see* **Note 1**).
3. Isonicotinic acid hydrazide (INH) (*see* **Note 2** and Subheading 2.4).
4. 10% v/v filter-sterilized glycerol (*see* Subheading 2.4).
5. Growth medium: LB broth, prewarmed to 30°C (*see* Subheading 2.5).

2.2 Electroporation of Mycobacterial Cells with EZ-Tn5 < R6Kγori/KAN-2 > Tnp Transposome and Confirmation of Transposition

1. Electrocompetent mycobacterial cells.
2. EZ::TN5 < R6K*γori*/KAN-2 > Tnp Transposome kit™ (Epicenter Biotechnologies, Madison, WI).
3. BTX electroporator (ECM 630; Harvard Apparatus, Holliston, MA).
4. Electroporation cuvettes: 0.1 cm electrode spacing cuvette (Bio-Rad, Hercules, CA).
5. Primers: KAN-2 FP-1 (5′-ACCTACAACAAAGCTCTCA TCAACC-3′) and R6KAN-2 RP-1 (5′-CTACCCTGTGGA ACACCTACATCT-3′), provided by EZ::TN5 < R6K*γori*/ KAN-2 > Tnp Transposome kit (Epicenter Biotechnologies).
6. Ex *Taq* polymerase (Takara, Madison, WI).
7. Lysis buffer (*see* Subheading 2.4).
8. Antibiotics: kanamycin and cycloheximide (*see* Subheading 2.4).
9. Growth media: 7H10 broth with OADC enrichment (Remel) containing 0.05% Tween 80 and 7H10 agar containing 25 μg/mL of kanamycin and 100 μg/mL of cycloheximide (*see* Subheading 2.5).
10. 96-well tissue culture microplates (Fisher Scientific).

2.3 Phenotypic Screening Using a PAH Spray Plate Method

1. 96-pin microplate replicator (Fisher Scientific).
2. Adhesive films for microplates (AlumaSeal96™ film, Sigma-Aldrich).
3. Sprayer containing 1% PAHs dissolved in acetone (*see* **Note 3** and Subheading 2.4).
4. PAH substrates of interest (*see* **Note 3** and Subheading 2.4).
5. Antibiotics: kanamycin and cycloheximide (*see* Subheading 2.4).

6. Growth media: LB broth and 150 mm diameter 7H10 agar plates supplemented with OADC (Remel, *see* Subheading 2.5).

2.4 Determination of Transposon Integration Sites by Plasmid Rescue Cloning of EZ-Tn5 < R6Kγori/KAN-2 > Transposed Genomic DNA of a Mutant with Metabolic Discrepancy or Defects in PAH Degradation

1. TransforMax EC100D *pir*-116 electrocompetent *Escherichia coli* (Epicenter Biotechnologies).
2. Ultraclean microbial DNA isolation kit (MoBio Laboratories, Carlsbad, CA).
3. Restriction endonuclease: *Sac*II (Promega, Madison, WI).
4. T4 DNA ligase (Promega, Madison, WI).
5. SOC medium (*see* Subheading 2.4).
6. Primers: KAN-2 FP-1 (5′-ACCTACAACAAAGCTCTCATCAACC-3′) and R6KAN-2 RP-1 (5′-CTACCCTGTGGAACACCTACATCT-3′).
7. Ex *Taq* polymerase (Takara, Madison, WI).
8. 50 μL of lysis buffer (*see* Subheading 2.4).
9. QIAquick plasmid miniprep kit (Qiagen, Valencia, CA).
10. Antibiotic: kanamycin (*see* Subheading 2.4).
11. Growth medium: 150 mm diameter 7H10 agar plates with OADC enrichment (*see* Subheading 2.4).

2.5 General Buffers, Media, and Reagents

1. Lysis buffer: 1% Triton X-100, 20 m*M* Tris–HCl, pH 8.0, and 2 m*M* EDTA.
2. Isonicotinic acid hydrazide (isoniazid): 40 μg/mL, filter-sterilized.
3. Kanamycin: 25–50 μg/mL.
4. Cycloheximide: 100 μg/mL.
5. Glycerol: 10% in filter-sterilized water.
6. SOC medium: 20 g Bacto tryptone, 5 g Bacto yeast extract, 2 mL 5 *M* NaCl, 2.5 mL 1 *M* KCl, 10 mL 1 *M* $MgCl_2$, 10 mL 1 *M* $MgSO_4$, 20 mL 1 *M* glucose/L water.
7. PAH substrates (pyrene, phenanthrene, fluoranthene, fluorene): 1% (wt/vol) in acetone.
8. LB broth: 10 g Bacto tryptone, 5 g yeast extract, 10 g NaCl/L water.
9. Middlebrook 7H9 medium supplemented with OADC enrichment (Remel).

3 Methods

3.1 Preparation of Electrocompetent Mycobacterial Cells

1. Inoculate 20 mL of LB broth with a 7-day single colony of a PAH-degrading mycobacterium from a Middlebrook 7H10 agar plate (*see* **Note 4**).
2. Incubate the culture at 30°C for 3–5 days with vigorous aeration (250 rpm).
3. Inoculate 200 mL of prewarmed LB medium containing 0.05% Tween 80 with 20 mL of the mycobacterial culture and continue incubation at 30°C overnight (*see* **Note 5**).
4. When the OD_{600} of the culture reaches 0.6, add filter-sterilized INH (4 μg/mL) and incubate the culture for another 4 h (250 rpm) (*see* **Note 6**).
5. Incubate the culture flask on ice for 1.5 h (*see* **Note 7**).
6. Harvest the cells by centrifugation at 9,000*g* at 4°C for 20 min (*see* **Note 8**).
7. Wash the cells three times with ice-cold 10% glycerol. (Reduce the volume each time. For example, for a 200 mL culture, wash first with 50 mL, wash second with 20 mL, and wash a third time with 5 mL.)
8. Harvest the cells by centrifugation at 9,000*g* at 4°C for 20 min. Decant the supernatant and resuspend the pellet in 1/50 to 1/100 of the original culture volume of ice-cold 10% filter-sterilized glycerol (*see* **Note 9**).
9. To use the electrocompetent cells immediately, proceed directly to **Step 1** in Subheading 3.2 (*see* **Note 10**). For future use, the electrocompetent cells should be frozen and stored in aliquots at −70°C.
10. To use frozen electrocompetent cells, the aliquots of cells should be thawed on ice (*see* **Note 11**).

3.2 Electroporation of Mycobacterial Cells with EZ-Tn5 < R6Kγori/KAN-2 > Tnp Transposome and Confirmation of Transposition

1. Mix 1 μL of the EZ-Tn5 < R6K*γori*/KAN-2 > Tnp Transposome with 85 μL of electrocompetent mycobacterial cells and leave on ice for 10 min (*see* **Note 12**).
2. Set the electroporation apparatus (a BTX electroporator, ECM 630; Harvard Apparatus) to deliver an electrical pulse of 2.4 kV, 200 Ω, and 25 μF for a 4 to 6 ms pulse duration (*see* **Note 13**).
3. Pipette the EZ-Tn5 < R6K*γori*/KAN-2 > Tnp Transposome/mycobacterial suspension to a cold 0.1 cm electrode spacing electroporation cuvette (Bio-Rad) (*see* **Note 14**).

4. Place the cuvette in the electroporation chamber and deliver a pulse of electricity to the cells with the settings indicated above.
5. Immediately remove the electroporation cuvette and add 1 mL of 7H10 broth containing 0.05% Tween 80.
6. Transfer the cells to a polypropylene tube (e.g., 17 × 100 mm or 17 × 150 mm) and incubate at 30°C for 4 h (*see* **Note 15**).
7. Plate different volumes (up to 200 μL per 90 mm plate) of the electroporated cells onto Middlebrook 7H10 agar plates containing appropriate antibiotics (in this study, 25 μg/mL of kanamycin and 100 μg/mL of cycloheximide) (*see* **Note 16**).
8. Incubate plates at 30°C until colonies become visible; this will take 3–5 days. Pick the Tn5 mutant candidates onto 7H10 agar containing antibiotics and incubate at 30°C for another 3–5 days to confirm growth of the transformants.
9. To confirm transposition, randomly pick colonies using sterile toothpicks and deposit them in 50 μL of lysis buffer (1% Triton X-100, 20 m*M* Tris–HCl, pH 8.0, 2 m*M* EDTA) in 1.5 mL microtubes.
10. Boil the microtubes for 10 min to prepare genomic DNA from chosen colonies.
11. Do PCR using KAN-2 FP-1 (5′-ACCTACAACAAAGCTCT-CATCAACC-3′) and R6KAN-2 RP-1 (5′-CTACCCTGTG-GAACACCTACATCT-3′) primers (*see* **step 5** in Subheading 2.2). The PCR reaction conditions are as follows: a cycle of 95°C for 2 min; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min; and a final extension of 72°C for 5 min. The final reaction volume is 50 μL.

3.3 Phenotypic Screening Using a PAH Spray Plate Method

1. Transfer colonies of mutant candidates into 96-well microplates containing 110 μL of LB broth using sterile pipette tips or toothpicks.
2. Replicate the transformed mutants using a microplate replicator, on the surface of 150 mm diameter 7H10 agar plates containing antibiotics from 96-well microplates (*see* **Note 17**).
3. Incubate at 30°C for 7–10 days (*see* **Note 18**).
4. Spray with a 1% PAH solution and incubate for 2–3 days to recognize a clear zone or halo around colonies in the film of PAHs (*see* **Note 19**).
5. Take a picture of the plates and record the characteristics of the colonies in detail (*see* **Note 20**).

3.4 Determination of Transposon Integration Sites by Plasmid Rescue Cloning of EZ-Tn5 < R6Kγori/KAN-2 > Transposed Genomic DNA of a Mutant with Metabolic Discrepancy or Defects in PAH Degradation (See Note 21 for Direct Determination of Transposon Integration Sites Using DNA Sequence Analysis of PCR-Amplified Transposon-Target Junctions)

1. Prepare genomic DNA from a chosen mutant with metabolic discrepancy or defects in PAH degradation (*see* **Note 22**).
2. Fragment 1 μg of the genomic DNA by restriction endonuclease digestions (e.g., *Sac*II).
3. Self-ligate 0.1–1 μg of DNA using two units of T4 DNA ligase in a 10–20 μL total volume for 1 h at room temperature (*see* **Note 23**).
4. Terminate the reaction and inactivate the T4 DNA ligase by heating at 70°C for 10 min.
5. Electroporate electrocompetent *E. coli pir*-116 using 1–2 μL of the ligation mix (*see* **Note 24**).
6. Immediately after electroporation, recover the electroporated cells by adding 1 mL of SOC medium to the electroporation cuvette.
7. Pipette the medium/cells gently to mix.
8. Transfer to a tube and incubate on a 37°C shaker for 30–60 min to facilitate cell outgrowth.
9. Plate cells on LB agar containing 50 μg/mL of kanamycin.
10. Select Kan-resistant colonies overnight.
11. Purify plasmid DNA using a QIAquick plasmid mini pre-kit.
12. Determine the DNA sequence of rescue clones, using two primers, KAN-2 FP-1 and R6KAN-2 RP-1.
13. Identify the Tn5-insertion position, using BLAST from the bacterial genome sequence

4 Notes

1. There are more than 150 recognized host-dependent and free-living bacterial species assigned to *Mycobacterium* (http://www.bacterio.cict.fr/m/mycobacterium.html) with remarkable metabolic and physiologic versatility. In general, PAH-degrading mycobacteria are fast-growing and nonpathogenic free-living mycobacteria. Among many others, so far, whole genome sequence of about ten strains of environmental mycobacteria, capable of degrading PAHs and other organic hydrocarbons, such as alkanes, alkenes, and vinyl chlorides, are available, in addition to *M. vanbaalenii* PYR-1. These include *M. aromaticivorans* JS19b1, *M. chubuense* NBB4, *M. rhodesiae* JS60, *M. rhodesiae* NBB3, *M. gilvum* PYR-GCK, *M. gilvum* Spyr1, and *Mycobacterium* spp. JLS, KMS, and MCS [39–42]. A full list of them can be found at https://img.jgi.doe.gov. Phenotypic and genotypic features of the PAH-degrading mycobacteria are significantly different from those of the

host-dependent pathogenic strains. Therefore, the phenotypic and genotypic differences of PAH-degrading mycobacteria from the pathogenic strains (such as *M. tuberculosis*) should be considered in designing experimental strategies and optimizing experimental parameters. On the basis of the available genome sequences, the genome size of PAH-degrading mycobacteria is between ~5.7 and 6.4 Mb with an average of 6 Mb, which is larger than that of *M. tuberculosis* strains (average genome size, ~4.4 Mb).

2. In this protocol, INH is used for a cell wall degrading agent to increase transformation efficiency. *See* **step 4** in Subheading 3.1 for more information.
3. Handle with care and store PAH substrates in a dry place protected from light.
4. PAH-degrading mycobacteria, which are usually fast-growing and nonpathogenic, should be maintained in the laboratory by regular subculture on solid medium (Middlebrook 7H10 agar). The medium used for growth of PAH-degrading mycobacteria for electroporation is not important, and a variety of different recipes can be used. However, we prefer using Middlebrook 7H10 agar supplemented with Tween 80 and OADC, since the agar plates make colonies of environmental mycobacteria conspicuous, which are yellowish in color (*see* **Note 5** below).
5. Owing to the thick, waxy nature of the cell coats, mycobacterial cells, including PAH-degrading species, tend to clump together in culture. Therefore, the addition of 0.05% Tween-80, a non-ionic detergent, to medium reduces the amount of clumping and provides a more homogenous suspension of cells (*see* **Note 8** below).
6. In pretreatment of the mycobacterial cells in logarithmic growth phase with a commonly used cell wall degrading agent, INH (4 μg/mL) results in an increase of transformation efficiency but minimal loss of viability. The combination of INH and glycine could increase the transformation efficiency [43].
7. For fast-growing PAH-degrading mycobacterial species, incubation on ice for 1.5 h before harvesting also increases transformation efficiency [44]. Although longer incubations on ice result in reduced efficiency, probably owing to increased cell lysis, the possibility of arcing during pulse delivery is also greater. Therefore, incubation time on ice should be optimized for the PAH-degrading mycobacterial strain of interest.
8. Cultures of PAH-degrading mycobacteria become slimy, probably because of the thick, waxy cell wall, which is rich in mycolic acid-containing long fatty acids. As explained above, it makes

the cells attach to one another, particularly after a long period of culture. Therefore, comparatively longer times of centrifugation are recommended as for bacteria (9,000*g* for 20 min) since, after centrifugation, the pellets can easily flow away when the medium is decanted.

9. For the transformation of a transposon-containing plasmid, bacterial cultures can be collected, and cell pellets can be resuspended in ice-cold 10% glycerol with 1/10 of the original volume. If homologous recombination is needed, 1/50 of the volume can be used.
10. As explained above, use fresh electrocompetent cells to maximize the number of transformants.
11. For future use, electrocompetent cells can be frozen with 20% glycerol and stored in aliquots at −80°C. Avoid a defrost cycle for the highest transformation efficiency.
12. DNA concentration in a volume of cell suspension is critical for high transformation efficiency. The conductivity of the cell suspension can be altered by improper use for volumes of cell suspension and the concentration (or amount) of DNA. It is recommended to use DNA in a volume of less than 5 μL. Transformation efficiency is increased by high concentrations (1.5–3 μg) of DNA.
13. Proper pulse conditions: The use of a pulse controller with electroporation apparatus makes it possible to control the parallel resistance and the time constant. An optimum time constant that we have observed was 5 ms. Cell walls of mycobacteria are chemically and physically resistant, thus the cells are not easily lysed and survive the high voltage (2.4 kV).
14. A variety of electroporation devices and apparatus are available commercially to deliver a high-voltage electrical pulse. We identified 0.1 cm gap electroporation cuvettes as the best after trying many different kinds. The best transformation efficiency was achieved by pulses with 5 ms under the parameters (2.4 kV, 200 Ω, and 25 μF).
15. Dilution of cells immediately after the pulse is important. Cells should be diluted at least tenfold and incubated for at least several hours before plating for better survival of transformants. Since PAH-degrading mycobacterial strains usually are fast-growing, incubation times of 2–5 h are suitable.
16. When using the EZ::TN5 < R6Kγ*ori*/KAN-2 > Tnp Transposome in *M. vanbaalenii*, the transformation efficiency was an average of 1×10^2 to 2×10^2 colonies in each electroporation experiment and often reached over 3×10^2 colonies [14]. Thus, it would be necessary to carry out multiple electroporations to ensure saturation of a genome, considering that

the average genome size of free-living PAH-degrading mycobacteria is ~6 Mb (https://img.jgi.doe.gov).

17. Based on experimental strategy, make multiple plates. When spotting the mutant culture, check the center spacing on your plate very carefully, as this varies between manufacturers. Also, the location of the matrix (position of the A1 well) varies between manufacturers.
18. Some mutants can grow fast and contaminate neighboring colonies. Check the incubated plates for optimal colony size for the subsequent PAH spray experiment.
19. PAH substrates can be strategically selected based on chemical properties; for example, since the degradation of some PAHs can be indicated by a color change, the absence of color change, together with no clear zone, clearly indicates no ability of mutants to degrade the color-producing PAHs. Examine plates periodically for a clear zone or color change around the colony after 2–10 days of incubation.
20. A time-lapse record of the mutant plates helps us understand the mutation by visually showing changes that we cannot easily perceive. For example, color and size of the colonies and development of clear zone around the colonies can be dependent on incubation time. This information for the mutants is essential to design and conduct the subsequent experiments systematically.
21. To directly define the gene location of transposon integration site using PCR, a uniquely oriented transposon-specific primer is able to be coupled with an arbitrary primer to PCR-amplify one of the transposon-target boundaries, which is subsequently sequenced in order to identify target DNA immediately adjacent to the transposon end sequence [45].
22. It is recommended to serially grow the mutant chosen in a fresh agar plate and double-check the phenotypic consistency of the mutant.
23. The extent of ligation can be quickly monitored by running aliquots of the reaction, before and after addition of the T4 DNA ligase addition, on an agarose gel.
24. Electrocompetent *pir E. coli*: *E. coli* expressing the Π protein, e.g., TransforMax EC100D *pir* + (maintenance of plasmid at ~15 copies per cell) or TransforMax EC100D *pir*-116 Electrocompetent *E. coli* (maintenance of plasmid at ~250 copies per cell). Any *pir* + *E. coli* strains can be used as electrocompetent cells.

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Microcalorimetry as a General Technique to Characterize Ligand Binding

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Abstract

Life is based on the specific interaction of molecules and the study of molecular recognition is therefore of fundamental importance. A number of different techniques have been developed to characterize binding events. Amongst these is isothermal titration calorimetry (ITC) in which heat changes caused by ligand binding are monitored. ITC is becoming increasingly popular, since it has a number of advantages over alternative techniques. ITC does not involve the immobilization or the labelling of ligands and there are little restrictions as to the choice of the buffer system and the analysis temperature. Since heat generation is a generic feature of an interaction, almost all types of ligands can be analysed including small molecules, proteins, or ribonucleic acids. However, the major advantages reside in the amount of information obtained in a single experiment which permits the determination of the equilibrium binding constants, the changes in enthalpy (ΔH^o) and entropy (ΔS^o), as well as information on the binding stoichiometry. In this chapter we provide practical guidelines for the generation of high-quality ITC data giving particular emphasis to troubleshooting. We will illustrate several examples how ITC can be successfully used to study the binding of hydrocarbons to sensor proteins.

Keywords: Isothermal titration calorimetry, Ligand binding, Microcalorimetry, Molecular recognition, Sensing

1 Introduction

The molecular interaction of any two ligands causes heat changes, which can be either exothermic (heat release) or endothermic (heat consumption). Using isothermal titration calorimetry (ITC), these heat changes can be measured and subsequently used to thermodynamically characterize the binding process. The instrument consists of a sample cell into which one ligand is placed and an automatic injector syringe for the other ligand (Fig. 1). An ITC experiment consists in the injection of aliquots of syringe ligand into the sample cell ligand and heat changes are constantly measured (upper panels of Fig. 2). Ligand injection is typically followed by a 3–4 min period

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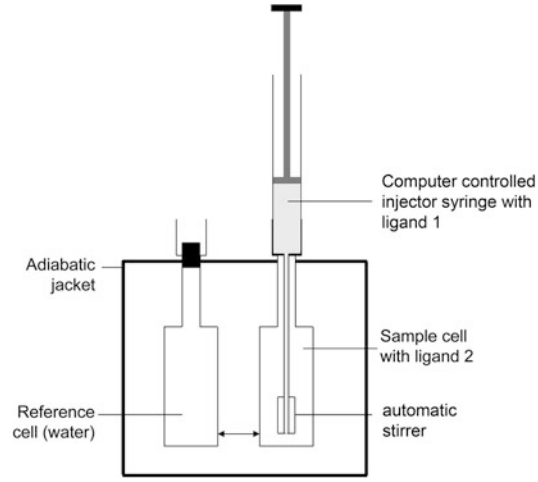

Fig. 1 Schematic representation of an adiabatic isothermal titration microcalorimeter. Heat changes in the sample cell with respect to the reference cell are measured

permitting the establishment of the equilibrium. In the design of an ITC experiment, it is important to achieve the saturation of the cell ligand with syringe ligand.

For data analysis, the raw titration data are integrated, corrected for dilution effects (typically the integrated peak areas of the dilution control are subtracted from the titration data), and normalized using the concentration of both ligands (lower panels of Fig. 2). These data are then submitted to curve fitting and in the cases shown a model for the binding of a ligand to a single site at a macromolecule was used. This procedure allows the determination of the association constant K_A and the enthalpy change ΔH^o. For sigmoid curves, K_A is related to the slope of the steep part of the curve (the steeper, the tighter is binding) and ΔH^o corresponds approximately to the point where the curve meets the *y*-axis. The dissociation constant K_D can then be determined using $K_A = 1/K_D$. The entropy change ΔS^o as well as the change in Gibbs free energy ΔG^o are calculated using

$$\Delta G^o = -RT \ln K_A = \Delta H^o - T\Delta S^o,$$

where R is the gas constant and T the absolute temperature.

The enthalpy and entropy changes determined provide very useful information on the nature of an interaction. This information, for example, permits the rational optimization of drug lead compounds [3, 4] which was traditionally based on trial and error approaches.

Figure 2 illustrates the use of ITC in the analysis of hydrocarbons. Part (a) of this figure shows titrations of the TodS sensor kinase with three hydrocarbons. The TodS/TodT two component

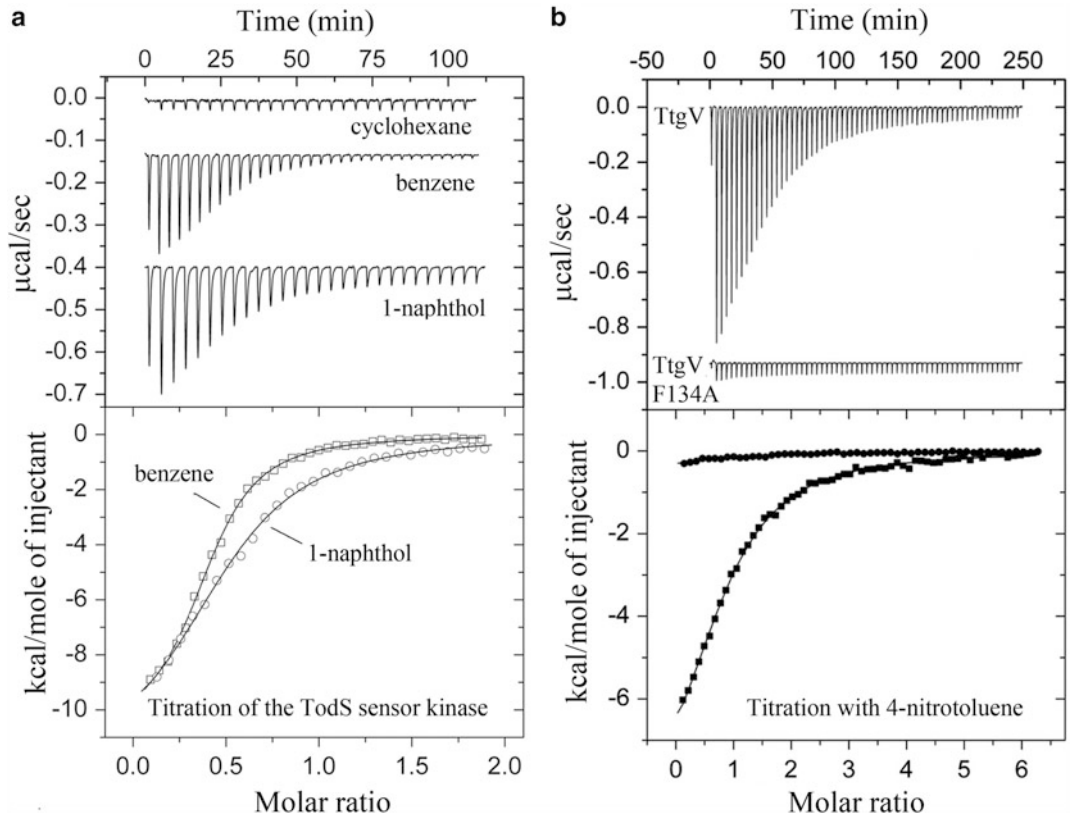

Fig. 2 Microcalorimetric titrations of transcriptional regulators with hydrocarbons. (**a**) Titration of 12–13 µM TodS sensor kinase with 500–750 µM cyclohexane, benzene, and 1-naphthol. (**b**) Titration of 35–37 µM of the transcriptional regulator TtgV and its site-directed mutant F134A with 1 mM 4-nitrotoluene. The upper panels show the titration raw data. The *lower panels* show the integrated, dilution-heat-corrected, and concentration-normalized peak areas of the raw data. The line corresponds to the fit with the "one binding site model" of the MicroCal version of Origin. Figures were taken from [1] and [2], respectively. These figure are reproduced with the permissions from the National Academy of Sciences, USA, Copyright (2007) and the American Society for Biochemistry and Molecular Biology, Copyright (2007)

system regulates the expression of the toluene dioxygenase pathway (*tod*) in *Pseudomonas putida* for the degradation of aromatic compounds [5], and we have used ITC to identify the hydrocarbons that bind to the TodS sensor domain [1, 6]. The heat changes observed for the titration of TodS with cyclohexane are small and uniform (Fig. 2a) and comparable to the titration of buffer with this ligand (not shown). Heat changes measured for the titration of TodS with cyclohexane are therefore due to ligand dilution and not due to binding, indicating that TodS does not recognize cyclohexane. In contrast, the titration of TodS with benzene and 1-naphthol gave rise to significant exothermic heat changes that diminished as protein saturation proceeded. Data analysis revealed that binding

was characterized by K_D values of 0.76 and 2.1 μM and enthalpy changes of −11.0 and −12.4 kcal/mol, respectively.

The TtgV transcriptional regulator controls the expression of hydrocarbon efflux pumps [7]. We were able to show that it exerts its regulatory activity following the recognition of different hydrocarbons of which some are substrates of the cognate efflux pumps [8]. Figure 2b shows an example of how ITC can be useful to determine the effect of site directed mutagenesis on hydrocarbon binding [2]. Shown is the titration of TtgV and its F134A mutant with 4-nitrotoluene. Binding to the wild-type protein was characterized by a K_D of 17 μM and an enthalpy change of −9.7 kcal/mol. Only small heat changes are observed for the mutant indicating that F134 is a central residue in ligand recognition.

Using TodS and TtgV as examples, we would also like to illustrate the versatility of ITC in the study of the regulatory systems. Titrations of recombinant TodS fragments and site-directed mutants with toluene have identified PAS1 as hydrocarbon-binding domain of TodS [1, 6]. Titration of TodS with TodT showed that both unphosphorylated proteins bind to each other with high affinity [9]. The titration of TodS with different hydrocarbons was the initial observation to identify agonistic and antagonistic compounds [1]. ITC titrations of TodT with various DNA promoter fragments have permitted to conclude that five TodT monomers bind to its promoter in a sequential and cooperative manner [10, 11]. The comparison of electrophoretic mobility shift assays and ITC experiments with different effectors allowed to conclude that there are different modes of hydrocarbon recognition at TtgV [2]. In addition, TtgV–DNA binding studies have identified the operator sequence of this tetrameric protein [12]. ITC was thus essential to elucidate the mechanisms by which the bacterium senses hydrocarbons and generates a regulatory response. Further information on general aspects and recent developments of ITC can be found in the following review articles [13–15].

2 Materials

1. Microcalorimeter.
2. Apparatus for sample degassing.
3. 2 ml gas tight loading syringe with 15 cm needle (http://www.hamiltoncompany.com/Syringes/, ref.:0161714).
4. 2 and 10 ml plastic syringes and 0.22 μm cutoff sterile filter units (http://www.eppendorf.com).
5. Dialysis membrane (http://www.spectrumlabs.com/).
6. 50 ml of dialysis buffer.
7. 2 ml of sample cell ligand.

8. 0.5 ml of syringe ligand.
9. Cleaning solution (Extran MA 03, www.merck.com, reference: 1.07550.2500).

3 Methods

3.1 Design of an ITC Experiment

3.1.1 Which Ligand Goes into Which Compartment?

The titration process is fully automatic and the input of the scientist is primarily required in the design of the experiment and data analysis. However, several issues need to be considered in the design of an experiment. In an ITC experiment one ligand needs to be placed into the sample cell and the other into the injector syringe. The user has thus to decide which ligand goes into which compartment. This choice depends on several factors. One has to consider possible nonspecific heat effects that may arise from the injection of the syringe ligand into buffer. Such effects have to be avoided since they hamper correct data analysis. Such undesired heat changes occur frequently when the syringe ligand dissociates upon injection. These heat changes seriously hamper or make a precise determination of binding parameters impossible. Dissociation is observed for protein multimers or compounds that form micelles such as gangliosides or lipopolysaccharides. To assess dilution effects, a control titration, ligand into buffer, is conducted. If heat changes are large, this ligand should be placed into the sample cell. Another factor to consider is the ligand solubility. The syringe ligand has to be present at a much higher concentration than the sample cell ligand. If one of the ligands has low solubility, it should be placed into the sample cell to be titrated with the ligand with higher solubility. The choice of which ligand goes where is also determined by the sample availability. One limitation of ITC is that comparatively large sample amounts are necessary. The sample cell ligand is saturated with an excess of syringe ligand which implies that larger amounts of syringe ligand are necessary to conduct an experiment. If the supply of one ligand is restricted, it should thus be placed into the sample cell. Sometimes a macromolecule contains multiple binding sites. To study ligand binding to a target with several binding sites, this ligand has to be placed into the sample cell. This is exemplified by the transferrin receptor which has two binding sites for transferrin [16]. When this receptor is titrated with transferrin, biphasic curves are observed from which the binding parameters of transferrin to both sites were determined. The inverse experiment, titration of transferrin with its receptor, results in a monophasic curve representing an average of both sites.

3.1.2 Making Sure the Sample Cell Ligand Is Saturated by Syringe Ligand

To obtain high-quality data, the sample cell ligand needs to be saturated with syringe ligand during the experiment. In cases where saturation is only partial, data can either not be analysed or the calculation of the parameters is subject to a considerable error. It is thus important to choose the right concentration for both ligands. Typically, the sample cell ligand concentration should not be significantly lower than 10 μM. However, two types of binding reaction can be distinguished. Firstly, an interaction where the K_D is significantly below the ligand concentration in the sample cell, which is the case for the example shown in Fig. 2a (titration of 12 μM TodS with benzene, $K_D = 0.76$ μM). This implies that the larger part of ligand injected binds to the sample cell ligand, which gives rise to sigmoid titration curves. To achieve saturation for this type of binding, a 2-fold molar excess of syringe ligand at the end of the experiment is sufficient for saturation. The second type of interaction is characterized by a K_D above or in the same range as the sample cell ligand concentration (Fig. 2b). In this case, a 2-fold molar excess of ligand will not cause saturation and higher syringe ligand concentrations are necessary to complete saturation. To design an ITC experiment, it is therefore desirable to have some approximate idea on the affinity. In the complete absence of such information, a relatively concentrated solution can be placed into the syringe. The first injection should be of a small volume (for instance, 2 μl). If the heat changes for this initial first injection are very small, which might but must not indicate weak binding, the injection volume can be increased for all subsequent injections.

3.1.3 Choice of Buffer

There are almost no limitations to the choice of buffer, and the primary factor is its physiological relevance and its capacity to stabilize molecules in their native states. Some binding processes are accompanied by proton transfer, which means that the molecular interaction causes the transfer of one or several protons from the ligands to the buffer or vice versa. Proton transfer involves buffer ionization or deionization which is not energy neutral and which contributes to the heat changes measured. This will distort the enthalpic contribution to binding but has no effect on the binding constant. To minimize an eventual contribution of proton transfer, a buffer system with a low ionization enthalpy should be chosen (phosphate, PIPES). Tris buffer has one of the highest ionization enthalpies and is therefore less suited. However, there is no problem in analysing an interaction which does not involve proton transfer in Tris buffer. To find out whether a binding process involves proton transfer, the experiment can be repeated in buffer systems with different ionization enthalpies. If the observed heat changes are comparable, no proton transfer occurs. To minimize dilution heats, both ligands have to be in the same buffer. This is best achieved by a dialysis of both ligands (in case both ligands are

macromolecules). If a macromolecule–small molecule interaction is studied, it is advisable to dialyse the macromolecule and to make up the ligand solution in dialysis buffer.

3.1.4 Signal-to-Noise Ratio Versus Number of Peaks

Each analytical technique is characterized by the signal-to-noise ratio. In an ITC analysis the signal-to-noise ratio can be optimized by injecting larger amounts of ligands. This, however, reduces the number of data points. In an optimal ITC experiment, there is a balance between the signal-to-noise ratio and the number of peaks. In general, some 15–20 data points are suitable to precisely determine binding parameters. Suboptimal data are characterized by an excessive amount of peaks which are small and therefore have a low signal-to-noise ratio. The other extreme, few but very large peaks, should also be avoided. The concentrations of both ligands as well as the injection volumes determine the number of peaks. Further information on the optimization of an ITC experiment can be found in [17].

3.2 Conduct of an ITC Experiment

3.2.1 The Procedure

1. Prior to the experiment the microcalorimeter is switched on and the required temperature is set, allowing the instrument to stabilize.
2. Verify proper functioning of the instrument doing a control titration (*see* **Note 1**). If the criteria for correct functioning (*see* **Note 1**) are not met, refer to **Notes 2** and **3**.
3. Around 50 ml of dialysis buffer and both samples are sterile filtered (exception: hydrophobic ligands which tend to stick to plastic surfaces). Both samples are degassed (exposed to vacuum) for around 5 min (exception: volatile compounds such as hydrocarbons). During these procedures the samples should be at the temperature of analysis.
4. The sample cell and the injector syringe are thoroughly rinsed with dialysis buffer.
5. Define experimental parameters in the software program (VPviewer): The experimental as well as injection parameters should be defined. Listed below are some commonly used parameters: reference power, 10 μcal/s; initial delay, 60 s (time from the beginning of experiment to first injection); stirring speed of injector, 300 rpm; feedback mode/gain, *high*; ITC equilibration options, activate *No check temperature* and *Fast equilibration*; injection volume, 3–15 μl; speed of injection, 0.5 μl/s; spacing between two injections, 240 s; filter period, 2 s (every 2 s a data point is recorded)
6. Sample cell loading: Fill the 2 ml loading syringe with the sample cell ligand. Insert the needle of this loading syringe into the sample cell until it touches the bottom and then lift it up some 3 mm. Inject the sample slowly but firmly. The last

300 μl should be injected rapidly to dislodge any bubbles. This last step should be repeated several times (*see* **Note 4**). The sample loading will destabilize the instrument; wait for around 10 min or until the signal is stable and close to zero (exception: if reducing agents are used, the signal will stabilize but will be offset into the exothermic range due to air oxidation).

7. Injector syringe loading: Fill the injector syringe with ligand by aspiration using a 2 ml plastic syringe connected via a rubber tube to the upper part of the injector syringe.
8. Introduce the injector syringe into the rotating mount of the instrument. The end of the syringe needle will be immersed into the sample cell solution. This will again destabilize the signal; wait for several minutes until the signal stabilizes (note: do not wait too long since syringe ligand will diffuse into the sample cell and vice versa; due to this reason the first injection is not used for data analysis).
9. Start of titration: The injector syringe is made to spin and the signal will approach gradually the reference power set in the main window. Once the signal is stable, the titration is started automatically.
10. End of titration: Once the defined sequence of injections has been completed, the titration stops automatically. Remove the injector syringe from the rotating mount. Remove the solution from the sample cell (*see* **Note 5**). Rinse sample cell and injector syringe several times with buffer and proceed with the next experiment.

3.2.2 Specific Issues Relevant to the Analysis of Hydrocarbons

The exact concentration of both ligands is necessary for the analysis of ITC data. Many hydrocarbons are volatile and evaporation reduces the ligand concentration in solution prior to its introduction into the instrument. The hydrocarbon solution should be made up in a closed recipient containing only a minimal amount of air. We recommend the preparation of ligand solution immediately prior to its introduction into the instrument. Typically, hydrocarbons are diluted into buffer, which is then vortexed for 10 s. After visual inspection to verify complete dissolution the solution is introduced into the instrument. Frequently hydrocarbon solutions can't be prepared at concentrations necessary for the syringe ligand. One option is to place the hydrocarbon into the cell and titrate with the other ligand. Another option is to prepare ligand stock solutions in dimethyl sulfoxide (DMSO) and then dilute the ligand 10-fold into buffer. The corresponding amount of DMSO (10 %, [v/v]) needs to be added to the other ligand solution. This is a strategy frequently used to study drug binding to target molecules [18]. Hydrocarbons tend to stick to surfaces. The use of glass vessels instead of plastics is recommended. As mentioned above,

the filtering and degassing steps are omitted. After a series of ITC experiments with hydrocarbons, it is recommended to clean the instrument with methanol.

3.2.3 Controls

As mentioned above the titration of ligand into buffer is necessary to validate the experimental data and a requisite for data analysis. It is advisable to use information available on the physiology of a macromolecule to identify adequate experimental conditions for ITC experiments. In this context we wish to cite the TtgV transcriptional repressor as an example. Genetic and microbiological data indicate that this protein binds specifically to two promoters and its physiological role appears to be restricted to the transcriptional regulation of the two corresponding operons [19]. There is no evidence that this protein has general DNA-binding properties or a global function. We were surprised to find that TtgV binds with high affinity to nonspecific DNA in buffer systems containing as much as 220 mM salt. Prior to the study of the interaction of TtgV with its target promoter, we had to identify experimental conditions which guarantees that TtgV binds specifically to its target promoter and not to nonspecific DNA [12]. Nonspecific DNA binding was fully suppressed by the inclusion of 300 mM salt into the analysis buffer.

At times, the microcalorimetric titration does not generate any significant heat changes. This may indicate that there is no binding, which however does have to be necessarily the case. It may be possible that at a given temperature, exothermic and endothermic contributions to binding cancel out each other and as a result no net heat changes are measured. Therefore, to conclude that there is no binding, the experiment has to be repeated at different analysis temperatures (note: the ratio of exothermic to endothermic contributions varies in function of the temperature). Only in cases where no binding heat changes are measured at two different analysis temperatures, it can be concluded that there is no binding.

3.2.4 Data Analysis

There are many ways to analyse raw titration data, of which one is by the use of the MicroCal version of Origin. The initial step in data analysis involves the manual optimization of the automatically generated baseline in order to correctly integrate all peaks. The exact concentration of both ligands is entered and the integrated peak areas of control data (ligand into buffer) are subtracted from the corresponding titration data. These corrected and normalized peak areas are then submitted to a fitting procedure. In general, a binding interaction can involve either a single or multiple events (e.g., when the ligand in the cell has several binding sites). Available biochemical data that provide insight into the number of binding events are most useful for correct data interpretation. Simple molecular interactions (one binding event) give rise to monophasic

curves (either sigmoid or hyperbolic curves) which can be fitted with the "one binding site model" of the Origin program (Fig. 2). Typically, more complex interactions (several binding events) give rise to more complex titration curves. Biphasic curves could be representative of two different binding events, which can either be independent or dependent (positive or negative cooperativity). The Origin software contains a model for the analysis of data with two independent events. In cases where the events are dependent, these data cannot be fitted with the model provided by Origin. Data can be analysed using either tailor-made models [20] or other programs of which we recommend the use of the SEDPHAT software package [21].

4 Notes

1. The best way to verify the proper functioning of the instrument is a titration of water with water (10 μl injections). The peak size should not be higher than 0.03 μcal/s and the baseline noise lower than 0.003 μcal/s. A gentle baseline drift is impossible to avoid but unproblematic.
2. The reason for a noisy baseline is in most of the cases a dirty sample cell which should be cleaned using either 10% SDS or a 5% solution of Extran MA 03 (Merck, reference: 1.07550.2500). Fill sample cell with the cleaning solution and leave at 25°C for half an hour. Then rinse thoroughly with water and do a water-into-water titration. If this control is still not satisfactory, repeat the cleaning procedure but incubate with cleaning solution at 65°C for half an hour, let it cool down, and rinse. This is likely to resolve the problem, but several hours are necessary to stabilize the instrument at the desired analysis temperature. As good laboratory practice a weekly cleaning of the instrument is recommended.
3. The reason for large dilution heats might be the distance of the injector needle with respect to the bottom of the sample cell, which is a very critical parameter. With time this distance changes and should be readjusted to 3 mm of the needle end above the cell bottom. Spikes in an ITC trace are either caused by air bubbles or unstable power supply. Samples should, if possible, be degassed and it is advisable to connect the instrument to a power stabilizer.
4. In some cases delicate proteins precipitate on sample loading into the syringe. During loading sample has to pass through the narrow hole of the syringe needle which represents a mechanical stress. Precipitation can be avoided by a very slow loading of the sample.

5. After the termination of a titration, the sample cell content should be inspected for precipitation which is sometimes induced by the molecular interaction. In this case heat changes caused by aggregation distort the binding enthalpy. Sometimes, a change in the buffer system prevents precipitation.

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