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Hydrocarbon and Lipid Microbiology Protocols

Isolation and Cultivation

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Editors

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Isolation and Cultivation

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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,

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

systems and synthetic biology tool usage, diverse applications, and 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
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Palma de Mallorca, Spain — Balbina Nogales

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Contents

Introduction to the Isolation and Cultivation of Microbes Involved in the Hydrocarbon Cycle 1
Terry J. McGenity

Protocols for High-Throughput Isolation and Cultivation 27
Karsten Zengler

Single Bacteria Studies Using Microfluidics 37
Yanqing Song, Bing Li, Yong Qiu, and Huabing Yin

Cultivating Fastidious Microbes 57
J. Cameron Thrash, Jessica Lee Weckhorst, and David M. Pitre

Cultivation and Preservation of Hydrocarbonoclastic Microorganisms, Particularly *Cycloclasticus* Species 79
Maria Genovese, Renata Denaro, Daniela Russo, Francesca Crisafi, Santina Santisi, Simone Cappello, Laura Giuliano, and Michail M. Yakimov

Cultivating Aerobic Hydrocarbon-Degrading Bacteria from Micro-algae 95
Tony Gutierrez

Two-Phase Cultivation Techniques for Hydrocarbon-Degrading Microorganisms . . . 107
Lukas Y. Wick, Sally Otto, and Christof Holliger

Protocol for Isolation, Screening, and Cultivation of Asphaltene-Degrading Microorganism 119
Seyed Morteza Zamir, Tina Tavassoli, and Seyed Abbas Shojaosadati

Cultivation of Anaerobic Microorganisms with Hydrocarbons as Growth Substrates 131
F. Widdel

Enrichment and Isolation of Metal Respiring Hydrocarbon Oxidizers 143
Adam J. Williamson and John D. Coates

Enrichment and Isolation of Chloroxyanion-Respiring Hydrocarbon Oxidizers 165
Tyler P. Barnum and John D. Coates

Cultivation of Methanogens 177
Taiki Katayama and Yoichi Kamagata

Cultivation of Methylotrophs 197
Donovan P. Kelly, Julie K. Ardley, and Ann P. Wood

Cultivation of Methanotrophs 231
Svetlana N. Dedysh and Peter F. Dunfield

Cultivation of Environmental Bacterial Communities as Multispecies Biofilms 249
Marc A. Demeter, Joe Lemire, Susanne Golby, Monika Schwering, Howard Ceri, and Raymond J. Turner

Cultivation of Hydrocarbon-Degrading Fungi 269
Dietmar Schlosser and Lukas Y. Wick

Oleaginous Microalgae Isolation and Screening for Lipid Productivity Using a Standard Protocol 283
Van Thang Duong, Boer Bao, and Peer M. Schenk

Preservation of Microbial Pure Cultures and Mixed Communities 299
Bram Vekeman and Kim Heylen

Erratum to: Protocols for High-Throughput Isolation and Cultivation 317
Karsten Zengler

Erratum to: Cultivation and Preservation of Hydrocarbonoclastic Microorganisms, Particularly Cycloclasticus Species 319
Maria Genovese, Renata Denaro, Daniela Russo, Francesca Crisafi, Santina Santisi, Simone Cappello, Laura Giuliano, and Michail M. Yakimov

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 to the Isolation and Cultivation of Microbes Involved in the Hydrocarbon Cycle

Terry J. McGenity

Abstract

Our knowledge of the uncultivated microbial majority is driving innovative approaches to cultivation, alongside novel cultivation-independent methods, to better understand microbial ecophysiology, biochemistry, and evolution. Biotechnological applications also provide a major impetus for obtaining microbial cultures, particularly in the field of hydrocarbon and lipid cycling, which address important societal challenges, including: biofuel production, anaerobic digestion, bioplastic synthesis, and mitigating the effects of diverse hydrocarbon pollutants and climate-active gases. This chapter provides a brief overview of cultivation strategies, focussing on those approaches that are most relevant to hydrocarbon cycling. The areas covered include: sample handling, strategies for delivery of carbon and energy sources and terminal electron acceptors, consideration of the physicochemical environment, supply of metabolites and undefined environmental components, assays for microbes, and preservation of cultures.

Keywords: Crude oil, Enrichment culture, Hydrocarbonoclastic, Microorganism

1 Background and General Strategies for Isolation and Cultivation

Microbes vary enormously in their capacity to grow on agar plates – some microbial weeds will grow rapidly on a variety of media designed for most organoheterotrophs, whilst the majority of microbes are much more fastidious and difficult to cultivate. Frequently, it is the latter that are the most abundant or active in situ. However, this is not always the case – in some environments, such as evaporator ponds in salterns [1], many of the abundant species identified by metagenetic or metagenomic approaches have been cultivated. Also, we have in culture some of the most widespread and abundant aerobic marine hydrocarbon-degrading bacteria, such as *Alcanivorax* and *Cycloclasticus* species [2, 3], which degrade alkanes and polycyclic aromatic hydrocarbons, respectively. Microbiologists are rejecting the idea that some (or even the majority of) microbes are "unculturable", referring to them instead as "uncultivated" or more optimistically "yet-to-be cultivated". Such optimism is

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justified by the isolation of microbes from taxa that previously had no cultivated representatives – the "microbial dark matter". For example, an epibiotic/parasitic isolate from the phylum TM7, which is detected in many environments across the globe, was recently described [4]. Moreover, there is likely to be much more functional diversity to be discovered in this broad phylum, with stable-isotope probing studies providing evidence that TM7-microbes can degrade monoaromatic hydrocarbons (e.g. [5]).

Despite the availability of many techniques to study microbes without growing them in the laboratory, it is still important to cultivate them. Pure cultures allow unambiguous data to be obtained about an organism's genome, its biochemical make-up, physiological potential, and phenotypic flexibility over a wide range of controlled environmental conditions. Isolated microbes may be characterised and described as new strains within a currently described species or as representatives of new species, genera, or even higher taxa, thereby widening our understanding of microbial diversity and evolution. Characterised strains are relatively stable (but *see* section on preservation) and are made available to the wider scientific community and so, unlike enrichment cultures or environmental samples, may be more easily subjected to reinvestigation and diverse forms of analysis. Although the genes of uncultivated organisms can be harnessed for biotechnological applications via metagenomic approaches, pure cultures can be easily manipulated, grown to high biomass and the link between genes and their function more readily explored. These features of pure cultures provide many possibilities for exploitation, for example, several hydrocarbonoclastic bacteria, are being investigated for the production of bioplastics, e.g. in the form of plyhydroxyalkanoates [6].

A combination of innovative cultivation approaches and careful screening is required to isolate new microbes that degrade or produce hydrocarbons or lipids, including many of those that have been identified as interesting and abundant species using culture-independent methods. This chapter should serve as a roadmap when trying to isolate and cultivate microbes (primarily of value to the newcomer), and whilst the focus is on those microbes involved in hydrocarbon or lipid cycling, many of the principles hold for any microbe. The chapter is far from comprehensive, as many of the details are provided in the other chapters in this volume. Also, valuable resources on microbial cultivation exist already. The series entitled *The Prokaryotes* provides excellent detailed information about cultivating all sorts of microbes. In particular, the reader should consult the comprehensive chapter in the 4th edition of *The Prokaryotes* entitled "Principles of Enrichment, Isolation, Cultivation, and Preservation of Prokaryotes" [7], which has developed from chapters in previous editions. Also, Tanner [8] provides a broad overview of procedures for cultivating a wide range of microbes.

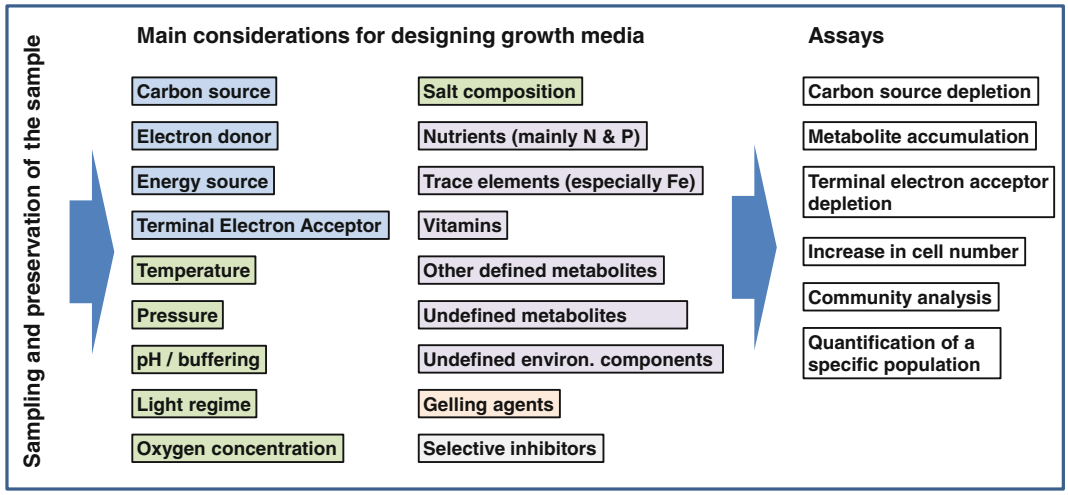

Fig. 1 *Roadmap of considerations when designing a programme for microbial enrichment, cultivation, and isolation*. The considerations are colour coded to represent logical categories (mostly corresponding with where they are discussed in the text). *Blue* – core metabolic considerations. *Green* – the main physicochemical conditions. *Purple* – other nutritional requirements. Other defined metabolites = cyclic AMP, homoserine lactones, etc. Undefined metabolites = extracts from other organisms. Undefined environ. components = extracts from the environment (usually in which the target microbe lives). Many other compounds may need to be added to the cultivation media, including indicator dyes (pH or redox) and reducing agents (for anaerobes). See the text for full details

Figure 1 provides some of the key considerations that will be discussed in more detail, but the overall aim of cultivation will determine the strategy that is used and so should be considered first. Examples of project aims are as follows:

Aim 1. Growing a pure culture that has already been described in the literature – This generally requires adopting a published medium, details of which can be obtained from original publications or culture collections, such as the DSMZ (https://www.dsmz.de). Nevertheless, if the strain of interest is going to form the mainstay of your research, it is worthwhile investing effort into optimising growth conditions.

Aim 2. Enrichment for microbes with predefined properties – The driver for this type of study might be to cultivate a microbe with the capacity to grow under particular physicochemical conditions and/or produce or consume certain compounds. In these cases, the medium and incubation conditions should reflect the desired properties, and/or the compound to be degraded should be provided as the sole carbon and energy source (or sometimes as sole N or S source, e.g. when strains are required for biodenitrogenation or biodesulphurisation [9]). When the aim is to isolate and cultivate microbes producing a particular metabolite, the screening process is of central importance, although the medium can also be manipulated in order to encourage the desired producers.

Such studies may have a biotechnological angle, and so there does not have to be a correspondence between desired function and the environment to be sampled (although the chances of success should be higher if they do correspond). For example, it is known that microbes with functions or tolerances that would not seem to be beneficial in a particular environment are frequently detected in that environment, either because microbes have been passively transported there (e.g. spore-forming thermophiles in cold-water sediments [10]), or the environment is more heterogeneous than bulk measurements suggest (e.g. alkaliphiles in soil of neutral pH [11]). It is important to consider that hydrocarbon-degrading microbes are found in low abundance in environments where there is no obvious external source of hydrocarbon contamination and are likely to be present wherever other life is found because many organisms produce and release a vast array of hydrocarbons. Such studies of seemingly out-of-place microbes can be valuable when addressing questions about their biogeography and dispersal, even exposing locally rare microbes that would not be detected by deep sequencing.

Aim 3. Enrichment of frequently abundant, but as yet uncultivated, microbes in a particular environment – This is generally the most difficult strategy, because there may be little background knowledge about the particular microbe. Therefore, it is usual to use many different types of media coupled with an assay to detect an increase in abundance of the desired microbe. Where possible, inferred knowledge about the physiology of the uncultivated taxon should direct cultivation efforts, for example, using approaches such as: (1) metagenomics (e.g. where functional and phylogenetic genes can be linked, either when they are found on the same fosmid clone or when the high concentration of cells of the species allows a genome to be constructed), (2) single-cell genomics, (3) correlation of species abundance with environmental data, (4) methods such as stable-isotope probing or MAR-FISH that link phylogeny with a specific function, and (5) clues from related species.

2 Sampling and Preservation of Samples

The sampling protocol can be as simple as scooping soil from your garden or as complex and expensive as drilling several hundred metres into sediments beneath the seafloor. Whatever the nature of the sample is, it is important to use sterile equipment and sampling technique. Ideally, sampling equipment should be autoclaved or gamma irradiated, but it is not always feasible to do this in the

field, in which case suitable disinfection regimes, e.g. using hypochlorite, should be employed. It is also essential to avoid cross contamination of samples, particularly when addressing questions about the tenacity of life. Specifically, it is important to be certain that microbes cultivated from remote, isolated, and low-biomass habitats truly derive from that location, for example, by rigorous testing of the sampling/drilling equipment in the case of subglacial lakes [12] and post-sample surface sterilisation in this case of buried salt [13]. For drilling into the deep subsurface, where the drilling fluid has an high abundance of microbes, tracers can be used to test the extent to which the core sample has been contaminated, which in turn will direct the sub-sampling from the sediment core [14].

Most obligate anaerobes are sensitive to oxygen, and so for their cultivation great care must be taken to avoid the ingress of oxygen into the sample. Aqueous samples with low concentrations of organic matter are more susceptible than organic- and clay-rich sediments, so the sampling/storage strategy should be appropriate for the sample. For example, in the former case, sampling bottles should be pre-flushed with nitrogen and a reductant added (*see* [15]), whilst in the latter case samples are more forgiving owing to slow oxygen diffusion and oxygen-consuming facultative aerobes and organic matter. Nevertheless, precautions should be taken, e.g. storage in anaerobic jars or wine bags [16] and transfer to an anaerobic cabinet as soon as possible [17, 18].

In order to maximise the chances of survival and viability of target microbes, certain other post-sampling physicochemical changes should be minimised, e.g. absence of light for obligate phototrophs, presence of light for chemoorganoheterotrophs from dark environments, and decreased pressure for piezophiles. A common post-sampling perturbation is a change in temperature; generally samples should be kept at in-situ temperature. Depending on the length of storage time required, it may be appropriate to slow down microbial activity by cooling the sample whilst avoiding freezing it. It is preferable, however, to set up enrichments as soon as possible after sampling.

Multiple samples should always be taken where possible, in order to be able to replicate interesting findings obtained with an initial sample, and to be able to underpin conclusions with robust statistical analysis. The researcher may also consider supplementing attempts to isolate and cultivate microbes from the habitat of interest with quantitative data, such as viable counts or most-probable-number analysis, coupled with questions about temporal or spatial variation, e.g. across environmental gradients. In such cases, replication must be consistent with the heterogeneity of the environment and the downstream statistical analyses. Environmental metadata are valuable when later investigating the ecology and biogeography of microbial isolates, and so a detailed description of the environment should be obtained, including time and date of

sampling, location, physical and chemical properties, as well as data on all associated biota (including microbes analysed by metagenetics or other approaches).

3 Carbon and Energy Sources

The majority of cultivated hydrocarbon-degrading species are aerobes, particularly those growing on structurally simple, low-molecular-weight hydrocarbons under mesophilic, nutrient-rich conditions. This is a consequence of a number of factors, such as: (1) alkanes and mono- and polycyclic aromatic hydrocarbons are the most abundant components of crude oil and are relatively rapidly biodegraded, (2) they are available as pure compounds or simple mixtures, and (3) their biodegradation is easily assayed. Thus, as well as being important compounds to investigate, low-molecular-weight hydrocarbons generally offer high reward for the researcher, in the sense that something will usually grow quickly. Nevertheless, there are regular additions to the diversity of cultivated aerobic hydrocarbon-degrading bacteria (e.g. by targeting new sources, such as algae-associated bacteria [19]), and there are many widespread and often abundant phylotypes that have evaded cultivation or have been difficult to maintain in culture, such as phylotypes related to Oceanospirillales ME113 [20]. In fact, *Cycloclasticus* species have proven difficult to maintain in culture, and Genovese et al. [21] provide helpful tips to obtain and retain pure cultures of members of this genus. Moreover, it is important to reconsider the roles of some of the generalists. For example, certain widespread marine species of *Pseudoalteromonas* are important in hydrocarbon degradation in the water column [22], and *Colwellia* was not widely considered as a genus with a major role in degradation of low-molecular-weight alkanes until the Deepwater Horizon oil spill in the Gulf of Mexico [23].

Methane is the simplest and probably the most important alkane, as it is an abundantly produced, potent greenhouse gas and an important fuel. Therefore, it is unsurprising that we have several dozen validly described species of methanotrophs in pure culture, as well as an in-depth understanding of their biochemistry, physiology, and ecology [24]. Despite this extensive understanding, new methanotrophs have been discovered relatively recently (e.g. acidophilic *Verrucomicrobia*), whilst other key groups are evading cultivation (e.g. candidate division NC10 that inhabit anoxic environments) [25]. Methanotrophs are considered to be nutritionally specialised. In contrast, most non-methanotrophic methylotrophs (i.e. those that grow with other C_1 substrates as well as compounds with multiple methyl groups) can also grow on substrates with C–C bonds [26]. However, a few known methanotrophs have the capacity to utilise compounds with more than one carbon atom, for example, *Methylocella sylvestris* can simultaneously

oxidise methane and other short-chain alkanes, such as propane [27]. Thus, it is important not to accept the dogma about any group of microbes, but to test their ability to grow on a wide variety of carbon and energy sources.

There are many thousands of different hydrocarbon structures found in nature – isoprene (2-methyl-1,3-butadiene), for example, is the structural subunit of terpenes that are oxygenated to terpenoids, of which there are thousands of examples. Moreover, isoprene itself is emitted into the atmosphere almost as abundantly as methane and has numerous impacts on climate. Nevertheless, this volatile, biologically produced, hydrocarbon has been the subject of only a handful of studies, with a relatively small number of isolates obtained (e.g. [28]).

Similarly, more attention needs to be given to microbes that specialise in degrading long-chain alkanes (e.g. > 30 carbons) and high-molecular-weight PAHs (e.g. > 4 benzene rings), for which there are relatively few cultivated representatives. Rosenberg [29] presents a number of approaches to enrich for microbes that degrade different fractions of oil, one of which involves the solvent extraction of hydrocarbons remaining after microbial growth on crude oil, resulting in crude oil that is depleted in the easy-to-degrade hydrocarbons, which then serve as the carbon and energy source for a new round of enrichment [30]. There is especially a dearth of studies on the use of asphaltene fractions of crude oil as a source of N, P, or C and energy. Improved methods to analyse the complex structure of asphaltenes should lead to developments in understanding their degradation, both by providing standard material to act as a feedstock and a means to assay biodegradation [31]. Similarly, we need an improved understanding of the diversity and degradability of oxygenated hydrocarbons, particularly naphthenic acids. They cause corrosion and form calcium precipitates that block and damage pipelines associated with oil exploitation and transport, and they are the major toxic component in large areas contaminated with oil-sands process-affected waters [32]. Also, oxygenated hydrocarbons accumulate and form de novo during crude-oil weathering [33] and are increasingly being transported globally as a major component of diluted bitumen (DilBit; [34]). Thus, there is an urgent need to investigate the fate of oxygen-containing hydrocarbons and the processes by which they are microbially degraded [35, 36].

As indicated in Aim 2 above, the strategy for cultivating microbes that produce hydrocarbons or lipids of interest relies on a combination of a suitable assay and appropriate carbon and energy sources. For example, if the product of interest is methane, then only those energy sources that can be used by methanogens (H_2, acetate, and some methylated compounds) should be provided, and the medium must be anaerobic with no added alternative terminal electron acceptors. Katayama and Kamagata [37] provide detailed protocols for the cultivation of these important Archaea and interestingly discuss coculture with a syntroph that provides a constant

supply of hydrogen at low concentrations, i.e. at levels seen in nature by most methanogens, an approach that led to the cultivation of *Methanocella paludicola*, a representative of the abundant and widespread phylotype, Rice Cluster I.

Duong et al. [38] describe the cultivation of oleaginous microalgae, which require light as a source of energy, and Morin et al. [39] describe cultivation of oleaginous yeasts. In both chapters, the focus is on the screening methods for the desired biotechnological properties as well as the means of encouraging lipid accumulation by controlling the C:N ratio in the medium.

4 Terminal Electron Acceptors

Oxygen may serve to activate hydrocarbons (catalysed by oxygenases) and as a terminal electron acceptor that results in high energetic gain to the cell. It is relatively straightforward to cultivate some hydrocarbon-degrading aerobes by simply providing a supply of air that can enter the culture vessel, e.g. via a nylon-wool plug. However, for volatile hydrocarbons the culture vessel needs to be hermetically sealed, in which case care must be taken to ensure that oxygen does not become limiting, for example, by retaining a large headspace-liquid ratio. In addition, a simple stoichiometric calculation should be performed to ensure that the vessel contains sufficient oxygen to allow degradation of the hydrocarbon at the concentration supplied, as illustrated in Box 1 (this simple calculation includes a number of assumptions and rounded numbers; for context *see* [40]).

Box 1. Oxygen available for benzene oxidation in a 120 ml serum bottle with 20 ml of minimal medium and how much benzene this can oxidise:
One mole of gas occupies a volume of about 24 l at room temperature.
Oxygen: air ratio ≈ 0.2

$$\text{Moles of } O_2 \text{ in serum bottle} = \frac{(\text{vol. bottle} - \text{vol. medium})\ \text{ml}}{24\ \text{l}} \times 10^{-3} \times 0.2$$

$$= \frac{120 - 20}{24} \times 10^{-3} \times 0.2$$

$$= 0.83 \times 10^{-3}\text{mole}$$

$$C_6H_6 + 7½\ O_2 \rightarrow 6\ CO_2 + 3\ H_2O$$

Benzene (C_6H_6) has a relative molecular mass of 78.

The oxygen present in each bottle can oxidise

$$= \frac{0.83}{7.5} \times 10^{-3} \times 78 = 8.63\ \text{mg benzene.}$$

This corresponds to 432 mg l^{-1} benzene.

In the example in Box 1, if benzene was added at 432 mg l^{-1} and was completely mineralised, then the oxygen concentration would reach zero during the degradation process, which may lead to a succession of microbial populations in an enrichment culture. This could be used to the researcher's advantage; alternatively, if oxygen depletion is to be avoided, then it should be monitored and the headspace refreshed periodically [41], or, for slightly volatile hydrocarbons, by growing with a continuous supply of oxygen and accepting and accounting for volatile loss by the use of uninoculated controls. Continuous culture provides a means to control delivery of volatile hydrocarbons and oxygen concentration [42].

An often neglected aspect of hydrocarbon degradation is microaerobic growth. Some of the possible scenarios by which hydrocarbon-degrading microbes may grow under low dissolved oxygen concentrations (<0.05 mg l^{-1}) are discussed by Aburto et al. [43] and include: microbes degrading the hydrocarbon by using trace oxygen as a reactant and terminal electron acceptor, use of trace oxygen as a reactant but not as a terminal electron acceptor, as well as interactions between coexisting aerobes and anaerobes. However, microaerobic conditions are rarely used for enrichment culture in a controlled way, despite low oxygen concentrations being a common feature of many environments. The cultivation of Acidobacteria was enhanced by incubations with low oxygen tensions, as well as elevated CO_2 concentrations, and addition of catalase and humic acids, demonstrating the value of such methods for studying this important soil phylum [44]. Many habitats have fluctuating oxygen concentrations, particularly intertidal flats, which combine tidal flooding (and thus reduced oxygen diffusion) with bioturbation that introduces oxygen to deeper sediments, and microalgal mats with diurnal changes in photosynthetic oxygen liberation. Such environments are also highly susceptible to oil pollution, motivating Terrisse et al. [45] to design bioreactors that simulate natural oxic-anoxic oscillations.

For the cultivation of obligately anaerobic microbes, and to be sure that observed activity is not a consequence of trace concentrations of oxygen, strict anoxic conditions are required. With the correct equipment (*see* [37, 46–49]), all manipulations can be performed on the laboratory bench. Anaerobic chambers are useful, e.g. for manipulating samples and when utilising agar plates, but cheaper anaerobic bags may also be used. Nevertheless, anaerobic cultivation requires investment in making or purchasing gassing manifolds and specialised glassware [46, 49], but it is worthwhile because our level of understanding of anaerobic hydrocarbon degradation lags behind that of aerobic degradation, and there are a greater diversity of mechanisms deployed to activate the hydrocarbon (and potentially more to be discovered) (*see* [50–52]), with important implications for oil-field operations and bioremediation.

All the nuances of anaerobic cultivation cannot be covered in this chapter, but are covered in detail in other chapters in this volume [37, 47, 48, 53] and include: (1) addition of the terminal electron acceptor of interest whilst avoiding other electron acceptors that may be present in the medium in other capacities, (2) delivery of diverse hydrocarbons whilst maintaining anoxic and sterile conditions, and (3) selection of reducing agents and redox indicators. The cultivation of (per)chlorate-respiring microbes, in which oxygen is produced transiently and likely activates hydrocarbons [54], is specifically discussed by Barnum and Coates [47].

5 The Physicochemical Environment and Nutritional Requirements

Incubation conditions and the composition of the medium should be governed by the aim of the research (see Aims 2 and 3 above), often reflecting in-situ conditions, but sometimes being modified to select for microbes with desired properties. Several points are considered below that should enter into the thought processes of medium design and incubation conditions.

Collectively, microbes occupy and grow within a huge array of habitats. Considering temperature alone, these habitats extend from sub-zero sea ice to boiling deep-sea vents. However, each species has a particular, restricted range of temperatures over which it can function, in terms of both width and position on the thermal scale. Now, if we consider another parameter, salinity for example, each species will occupy a particular window in temperature-salinity niche space. This notion can be extended into multidimensional niche space considering an array of physicochemical conditions. The two-dimensional niche window can be determined by measuring temperature growth range and salinity growth range when cultured under optimal conditions for the alternative parameter. However, this is a theoretical window, because a species' functional range for one physicochemical parameter is affected by other parameters, including other physicochemical conditions, biotic effects, carbon and energy sources, and electron acceptors. For example, for some marine strains of *Halomonas*, a salinity of 11% (compared with 4%) enhanced their capacity to grow at lower temperatures [55]. Therefore, it is important to control the physicochemical environment, considering multiple parameters, in order to cultivate the desired microbes. In this respect, the interplay between pressure and temperature for deep-sea methanogens is discussed by Tasumi et al. [56]. If the research aim is to cultivate a diverse range of microbes, then it is important to vary permutations of the environmental growth conditions (in addition to carbon and energy sources and terminal electron acceptors).

5.1 Temperature

The above points are illustrated by Coulon et al. [57], who showed that for Thames Estuary water (temperature at time of sampling 8°C, annual range 3–19°C) enriched with crude oil, incubation at 4°C and 12°C yielded similar microbial communities that were distinct from those grown at 20°C. Certain phylotypes (e.g. *Roseobacter* sp.) were found in similar abundance at both 4°C and 20°C, whereas others (e.g. *Oleispira* sp.) were found uniquely at 4°C. In addition to the direct effects of temperature on microbial physiology, it can alter the bioavailability of hydrocarbons as discussed by Coulon et al. [57]. Temperature also impacts on other physicochemical properties, which should be accounted for in cultivation studies, for example, pH is temperature-dependent and the solubility of oxygen in water decreases as the temperature increases.

For general considerations about cultivation, the following articles should be consulted: for thermophiles, Nakagawa and Takai [58], and for psychrophiles, Russell and Cowan [59] and Lo Giudice and Rizzo [60]. Given the slow growth rate at low temperature, the issue of overgrowth by weeds is exacerbated [59], and so the isolation-before-cultivation approaches of Thrash et al. [61] and Zengler and Behrendt [62] would prove valuable. Tasumi et al. [56] provide details for high-pressure, high-temperature incubation of the most thermophilic organism known, the hydrogenotrophic methanogen, *Methanopyrus kandleri* strain 116. In addition to methane formation in deep marine environments, it is important to consider the interacting factors that may limit microbial life in deep, hot oil reservoirs. Such an understanding would help to predict the susceptibility of reservoirs to souring, and representative sulphate-reducing, thermophilic isolates would be valuable in this regard. Also, given the spread of oil exploration and exploitation in cold regions of the Earth, especially the Arctic, it is important to understand the impact of (low) temperature on biodegradation rates [63] and to obtain model microbes in pure culture that can provide insights into the mechanisms of hydrocarbon degradation around 0°C.

5.2 Pressure

High pressure is the environmental parameter that requires most skill and specialised equipment to control; consequently it is often neglected in cultivation studies. The classic work of Yayanos [64] illustrates the range of pressure tolerances/requirements of marine microbes, with some deep-sea strains being able to grow to some extent at atmospheric pressure. However, it was also conclusively shown that some deep-sea microbes are obligately piezophilic [64]. By analysing many data sets, Tamburini et al. [65] conclude that incubations of deep-seawater at atmospheric pressure generally lead to lower microbial activities than at in-situ pressure. Therefore, a lot of the most important microbes in deep environments will be missed if samples are not collected and maintained under high pressure. The shallowest depth at which obligately piezophilic microbes are likely

to be found is not known, but below 500 m water depth, the use of high-pressure cultivation should be considered. Smedile et al. [66] explain the importance of understanding hydrocarbon degradation processes under high pressure in order to better predict the fate of oil after events like the Deepwater Horizon spill, and provide details of their pressure-retaining sampler and high-pressure incubator for the cultivation hydrocarbon-degrading microbes. For a range of other considerations when culturing microbes at high pressure, the review by Kato [67] should be consulted.

5.3 pH

Collectively, microbes can grow in the pH range from ~0 to 11, with individual species tending to grow within a range of ~1.5–5 pH units. From numerous environmental studies, pH comes out as the factor that appears to most greatly influence microbial community composition. Thus, pH is an important parameter to control. Moreover, products of microbial growth and atmospheric components, such as CO_2, may alter the pH of the medium, especially in low-nutrient minimal medium that is likely to be used to culture hydrocarbon-degrading microbes. Therefore, pH should be monitored during incubation, and buffers may be added to the medium to control pH over the desired range [7]. The buffers must be chosen judiciously, always considering whether they may be toxic or serve as a source of carbon, nitrogen, or phosphorous. This is especially important when culturing microbes from low-nutrient environments. Phosphate buffers are sometimes used by default, but excessive concentrations inhibit many microbes and can form a precipitate with other media components [7, 68]. For a discussion of hydrocarbon- and methane-oxidising microbes growing at the extremes of pH, the following should be consulted, acidophiles [69], alkaliphiles [70], and methanotrophs [25], whilst valuable tips for growing methanotrophs and methanogens from acidic wetlands are provided by Dedysh [71].

5.4 Light

When enriching for hydrocarbon-degrading microbes, it is normal to incubate in the dark. However, incubating in the light can result in a different microbial community composition, leading to the selective enrichment of novel hydrocarbon-degrading microbes. Such microbes may include hydrocarbon-degrading anoxygenic phototrophic bacteria [72], about which we know very little. In addition, light may benefit proteorhodopsin-containing bacteria, and particularly aerobic anoxygenic phototrophic bacteria, which use light to supplement chemoorganotrophic growth, which may include hydrocarbon degradation, e.g. *Roseobacter* clade bacteria. Light will allow growth of oxygenic phototrophic eukaryotic microalgae and cyanobacteria, which in turn will provide new organic matter to the system, which would benefit facultative hydrocarbon

degraders. Many phytoplankton species produce and export hydrocarbons. For example, global annual alkane production by marine cyanobacteria (primarily synthesising *n*-heptadecane) is orders of magnitude greater than the mass of hydrocarbons released by the Deepwater Horizon spill [73]. Microalgal hydrocarbons will add to the pool of hydrocarbons in the growth medium and may in turn alter the bioavailability of supplied hydrocarbons. Many facultative and obligate hydrocarbon-degrading bacteria live in close association with microalgae (probably as a consequence of microalgal hydrocarbon production), and so the coculture of phototrophs and heterotrophs will select for algae-associated strains [19, 74]. Light can also alter the structure of hydrocarbons. All of these factors show that light should be considered in the search for new hydrocarbon degraders. Care must be taken to select the intensity of photosynthetically active radiation that is appropriate to the microbes under investigation.

5.5 Major Ions and Salinity

Salinity has a major effect on microbial community composition (see [1]), and so it is surprising that it is sometimes inadequately considered when trying to grow representative microbes from the environment. The salinity or water activity of the environment should be measured, and, in order to minimise osmotic stress or lysis, the medium and any diluting solutions should be in osmotic equilibrium with the environmental sample. Analysis of the major ions will allow the design of a medium with a similar ionic composition to the environment. As with many physicochemical parameters, a snapshot measurement at the time of sampling is valuable, but a guide to cultivation strategy is better served by knowing how salinity varies over time and space. The surface of salt-marsh sediments, for example, may vary from almost freshwater salinity (after heavy rainfall) to the point of sodium chloride precipitation (~5 M NaCl; following a period of desiccation after tidal inundation). In contrast, variations in the salinity of oceanic surface waters are trivial. An example of a freshwater medium is provided by Bartscht et al. [68] and for seawater by Dyksterhouse et al. [2], but there are many variations on a theme. Some considerations when growing microbes from environments where the salinity exceeds that of seawater are provided by McGenity and Gramain [75].

5.6 Nutrients

The most common approach for bioremediation of hydrocarbon-polluted environments involves the addition of nutrients, primarily N and P, to provide the mass-ratio of C:N:P approximating that required for balanced cell growth [29]. However, for cultivation, it must be considered that an excess of the carbon source is frequently provided, perhaps because it is poorly bioavailable, and so rigidly following this ratio may lead to nutrient overload. It is suggested that the nutrient concentrations in growth media are considered carefully, balancing the properties and concentration of the

hydrocarbon and thus the required stoichiometry with the nature of the environment (and thus whether the microbial inhabitants are likely to be oligotrophic). Also, supplying N and P in different forms [29] may select for different microbes; and by omitting N completely, dinitrogen-fixing hydrocarbon-degrading bacteria may be selectively enriched. Important but simple steps can help the cultivation of microbes that are more representative of the in-situ environment, for example, autoclaving phosphate separately from agar or removing it from the medium to prevent the production of oxidising agents [76]. N and P are usually supplied in the form of salts, and for some freshwater microbes, the additions may be sufficient to make the medium osmotically suboptimal.

5.7 Trace Elements, Vitamins, and Other Growth Factors

Microbial trace-element requirements vary widely from species to species, and sometimes they may be adequately supplied as contaminants of other media components. Moreover, too much of a trace element can inhibit growth of some microbes. However, there are numerous reports of specific trace-element requirements, e.g. cobalt, nickel, iron, zinc, molybdenum, and/or tungsten for methanogens [37, 77]. The community composition in a methanogenic enrichment was found to be affected by the overall concentration of a trace-element mixture [78] as well as the relative concentrations of particular metals [77]. Such systematic studies of trace-element requirement are relatively common for methanogens because of the need to optimise and control their industrial activities, such as biogas production. Also, it is well known that methanotrophs are influenced by trace-metal additions to their media, because the particulate methane monooxygenase is a copper-containing enzyme [24]. However, I am not aware of systematic studies examining the trace-element requirements of most hydrocarbonoclastic bacteria. A variety of trace-element solutions have been used when enriching for hydrocarbon-degrading microbes (see chapters in this volume, e.g. [19, 21, 25, 26, 47, 48, 61]), and if a mixture of trace elements is not added, iron should be supplied.

Not all microbes have the capacity to synthesise all the vitamins they require for growth. In nature (and probably in enrichment cultures), such organisms will draw on the pool of vitamins that have leaked from other cells, but they cannot do this in pure culture. The addition of vitamins to growth media will also benefit organisms that do synthesise them, because it allows resources to be directed to other metabolic pathways. A filter-sterilised mixture of vitamins is therefore commonly added to cultivation media [19, 21, 25, 26, 47, 48, 61, 79]. The vitamin (and trace-element) requirements of cultivated hydrocarbon-degrading microbes are rarely investigated subsequently, and there are few reports examining how vitamin addition to minimal medium affects the community composition in an enrichment culture.

Some bacteria lack the capacity to synthesise certain amino acids (and even purines and pyrimidines), a fact that is not always accounted for in the design of enrichment media for hydrocarbon-degrading bacteria. To address this issue, a defined medium may be used, with the amino acids added as separated components or supplied as a complex mix, e.g. as Casamino acids or as components of yeast extract. Microbes may use the amino acids (and other components of yeast extract) as a source of carbon and energy in preference to hydrocarbons, thereby selecting against obligate hydrocarbon-degrading bacteria. Consequently, the concentration of the supplements must be as low as possible, and parallel enrichments should be performed with and without amino acids.

6 Counter-Selective Agents

Overmann [7] describes a number of counter-selective agents, but antibiotics are the most commonly employed in enrichment cultures, owing to their specificity. Examples include: cycloheximide to prevent overgrowth by fungi, ampicillin, and other antibacterial compounds to encourage growth of Eukarya and Archaea, and bacitracin to select against Archaea. An atypical base substitution was observed in 16S rRNA sequences of TM7, a microbial candidate division noted earlier for its putative benzene and toluene degradation, which should provide resistance to streptomycin [80], thus providing a potential means for its enrichment [4].

7 Gelling Agents: Alternatives to Agar

The polysaccharide, agar, is the default solidifying agent, but there are situations in which other compounds would be a better choice. In principle, anything with a pore size smaller than a bacterial cell may substitute agar. Gellan gum (commercial name, Gelrite) is frequently used for growth at high temperature. Deguchi [81] promotes the use of nanofibrous cellulose especially for certain extremophiles. Polycarbonate filters floating on liquid medium [82] have proved useful in culturing novel verrucomicrobial methanotrophs [83] and similar microbes that are susceptible to overgrowth by organisms that may grow on trace organics in agar. Walker and Colwell [84] showed that media solidified with silica compared with agar led to the growth of more hydrocarbon-degrading bacteria. Although some of these approaches are sometimes less convenient than using agar, it is important to consider alternatives, particularly for organisms that may be inhibited by agar or its impurities or outcompeted by other microbes that grow on the contaminants in agar or even the agar itself.

8 Design of Cultivation Equipment, Delivery of Hydrocarbons, and Supply of Metabolites and Undefined Environmental Components

The almost default procedure for growing aerobic microbes is to add an inoculum into the medium in a flask that is then shaken. Researchers interested in growing anaerobic microbes have had to be more inventive in their approach to cultivation and the design of their equipment (e.g. [37, 48]). However, there are many ways in which modifications to strategies and equipment can selectively enrich for, and allow enhanced cultivation of, novel and interesting microbes.

Hydrocarbons and related polar molecules vary widely in their physicochemical properties, from highly soluble gases to almost insoluble solids. Crude oils are also highly variable in composition and physical properties. Prince et al. [85] provide guidelines for preparing different fractions from crude oil, ranging from artificially weathered crude oils in which volatile compounds are removed to water-accommodated fractions that contain soluble oil components. Various refined products are also described. When hydrocarbon degradation is being used as an assay, it is important to consider the addition of a nondegradable marker compounds and also to practise accurately dispensing viscous or volatile oil fractions [85]. Given the diverse properties of crude oil, refined products, and individual hydrocarbons, innovative means of delivery are required to allow microbial access to the growth substrate whilst avoiding toxicity. A typical method for volatile hydrocarbons is to use a tube that passes tightly through a hole in the flask's PTFE-lined septum/lid. The tube is sealed at both ends but has a hole to allow vapours to escape into the headspace and partition into the liquid medium. Many of the protocols in this volume provide details of hydrocarbon supply, including the use of two-phase systems, e.g. adsorbed onto a solid matrix or dissolved in a nondegradable, non-toxic solvent phase [48, 85, 86]. Bucheli-Witschel and Egli [42] describe an approach for growing hydrocarbon-degrading microbes in continuous culture, which allows the investigation of degradation at low substrate concentrations and also provides a steady-state culture that is a prerequisite for certain physiological and gene-expression analyses.

When growing hydrocarbon-degrading microbes on solid media, care should be taken to include control mineral-salt agar plates with no added hydrocarbon, in order to ensure organic compounds in the atmosphere or agar does not support microbial growth [87]. It is recommended to wash the agar with acetone (followed by multiple rinses with water) or to use other gelling agents (see above). As with liquid cultures described above, specific properties of the hydrocarbon must be accounted for. Growth with volatile hydrocarbons can be performed by incubating plates in a hermetically sealed jar and placing the volatile compound in a dish within the jar or on filter paper placed

under the lid of the plates. Solid hydrocarbons can be delivered by dissolving them in a solvent such as acetone, filtering through a 0.2-μm solvent-resistant membrane, spraying onto plates, and allowing the solvent to evaporate, which leaves behind evenly distributed hydrocarbon crystals [88, 89]. This method may also be applied to liquid media, supplying solid hydrocarbons with an increased surface area compared with when the crystals are added directly. In all cases, solvent-only controls are required to check that residual solvent does not alter growth. The agarose overlay method also works well for ethanol-resistant strains [90]. The pros and cons of these techniques are discussed by Shuttleworth and Cerniglia [89], and an alternative solvent-free method that employs sublimation has been described by Alley and Brown [91]. Liquid hydrocarbons that are not very volatile may be spread directly onto the surface of plates, but distribution may be uneven. Rosenberg [29] describes the method of Colwell et al. [92], in which hydrocarbons or crude oil are dissolved in diethyl ether, mixed with silica, and autoclaved with the medium, in order to provide an even distribution. Walker and Colwell [84] showed that autoclaving the oil with the agar medium leads to no decrease in the number of hydrocarbon-degrading microbes obtained.

When using toxic, volatile hydrocarbons, manipulations must be performed in a fume extraction hood. Microbial contamination should be negligible as long as the hood is thoroughly cleaned and disinfected, gloves are disinfected, and bottle/plate opening times are minimised. It is important to consider how or whether the hydrocarbon of choice will be sterilised. For gaseous and liquid hydrocarbons, this is generally done by filtration, e.g. through a tortuous-path filter (e.g. nylon wool) or polytetrafluoroethylene (PTFE) membranes, respectively. But some solid hydrocarbons are not easy to sterilise (unless first dissolved as described above), in which case it is always important to use no-inoculum controls to ensure that the hydrocarbon itself is not a source of microbes. It is recommended to include this hydrocarbon-only control even if the hydrocarbon has been sterilised, especially for shaken liquid cultures, so that emulsification of the hydrocarbon caused by shaking can be differentiated from microbially induced emulsification.

Many microbes grow preferentially as a biofilm, and simply adding small glass beads to a liquid enrichment can alter the types of microbes enriched (personal observation). By adding hydrophobic filters containing PAHs to the culture medium, Bastiaens et al. [93] were able to enrich for and culture microbes, specifically *Mycobacterium* spp., that preferentially grew as a biofilm. Demeter et al. [79] indicate that the reason why many microbes have not been isolated is because they have obligate interactions with other microbes [74] and so focus on analysing mixed cultures. Specifically, they describe the Calgary Biofilm Device for cultivating and investigating attached microbes, recording cultivation (but not

isolation) of 75% of the original phylotypes detected in the inoculating sludge from an oil-sands tailings pond [35, 79].

The chapters by Thrash et al. [61] and Zengler and Behrendt [62] present innovative methods that have been used to cultivate a wide range of novel microbes including *Candidatus Pelagibacter ubique*, representing the SAR11 clade, probably the most abundant bacterial group in the ocean [94]. In contrast to the traditional approach of combining growth and isolation on agar plates, both methods employ cell separation before cultivation, using dilution coupled with separation in microtitre wells [61] or agarose microencapsulation [62]. These dilution-based approaches commonly add water from the environment under investigation to the growth medium and maintain low levels of nutrients and provide excellent means to cultivate abundant microbes that can grow well alone. It is, however, well established that many microbes grow less well or die after cultivation as a pure strain in the absence of other organisms [21, 35, 74, 95], and so dilution and pre-culture separation will not always be an appropriate strategy.

The interdependence of microbial species may manifest itself to the researcher trying to obtain pure cultures in several frustrating ways, such as: (1) the pure culture grows but then is not maintained in a viable state in the laboratory; (2) the seemingly pure culture grows, but after further subculture it turns out not to be pure or has been completely excluded by its latent coculture; (3) the desired microbe is in high abundance in enrichment cultures but cannot be obtained as a pure culture. Lagier et al. [96] and Stewart [95] reviewed several devices that allow diffusion of chemicals from the environment into the growth medium (or simply into the agar). Such devices [95, 97, 98], when coupled with microcolony selection, have proven to be very powerful in providing cultures of traditionally difficult-to-grow microbes. An array of growth-enhancing biochemicals added to media has also led to enhanced cultivation, including: cyclic AMP, homoserine lactones, catalase, siderophores, and signalling peptides. However, when two strains are difficult to isolate from each other, and grow better together, it is not trivial to identify the growth-enhancing metabolites; therefore the possibility of isolation and further growth as a pure culture may be improved by adding from the mixed culture (or a related pure strain): autoclaved cells, lysed cell filtrate or dialysate, or spent medium from cultivated cells, etc. Alternatively, one strain may be grown alongside the other in a system that allows metabolites but not cells to be transferred to the medium, e.g. via some form of filter or dialysis bag. One may speculate about the nature of these unknown growth-enhancing metabolites – they may act directly on the cell or enhance the bioavailability of oil. Biosurfactants are a good candidate for the latter, but have largely been overlooked as media components despite their capacity to increase the bioavailability of poorly soluble hydrocarbons [74]. Their value was

illustrated by Calvo et al. [99], who showed that a microbial extracellular polymeric substance with biosurfactant properties enhanced the cultivation of microbes growing on PAHs.

Advances in microfluidics [95, 100] provide the enabling technology to investigate single cells in a culture and so understand features of population heterogeneity. The protocol by Song et al. [100] describes the formation of a bacterial cell monolayer, which can be investigated microscopically after precise perturbations of the environment. It is exciting to envisage the many ways in which modified devices could be used to study cultivated hydrocarbon-degrading/producing bacteria and even be employed to encourage cultivation of new species.

9 Assays

Assays associated with enrichment cultures are many and varied but generally include some measure of degradation of the hydrocarbon provided as a growth substrate or as a co-metabolite using methods such as gas chromatography with flame ionisation detection [101, 102], alongside some form of quantification of the microbes of interest, for example, using real-time PCR [103] or specific molecular markers such as ergosterol for fungi [104]. Measurement of metabolites can indicate the extent to which a single hydrocarbon has been mineralised to CO_2, as well as providing insights into the mechanism of degradation. By additionally measuring changes in the concentration of the terminal electron acceptor and/or its reduced product [48], a mass balance of the reaction may be obtained and anaerobic degradation can be confirmed.

Beggah and Van der Meer [105] employed a procedure of cell staining and flow cytometry in order to quantify small increases in biomass that would not result in a turbid culture. Coupling such an assay to innovative enrichment techniques should allow the capture of microbes that grow to a low cell density, where the hydrocarbon of interest is supplied at low concentration (e.g. to cultivate microbes that preferentially utilise low concentrations of the hydrocarbon or to avoid adding too much of a toxic compound) or when the hydrocarbon is not very bioavailable. When assaying for growth, it is important to recall that microbes may grow as a biofilm, especially when supplied with poorly soluble hydrocarbons as a gas, liquid, or solid. In such cases, methods of microbial quantification may have to be modified, e.g. using imaging approaches [79] and/or cultures thoroughly dispersed prior to more standard quantification.

10 Preservation of Cultures

An apparent pure culture may harbour other microbes that are in low abundance and remain hidden from microscopic and molecular analyses. Although such organisms may not reach high cell abundance on the medium being employed, they may do so on other media. Therefore, it is advisable to check cultures on different media to ascertain their purity. Pure cultures must then be preserved because they consist of a heterogeneous population that will evolve in the artificial laboratory environment. Even a few generations of growth in the absence of the hydrocarbon that serves as a carbon and energy source may lead to the loss of function, e.g. by plasmid curing [106]. The typical method for preservation involves freezing at −80°C in the presence of a cryoprotectant, which is discussed by Genovese et al. [21] in the context of hydrocarbon-degrading microbes and in great detail by Vekeman and Heylen [107], who consider a broad range of preservation scenarios, including anaerobes and microbial consortia.

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Protocols for High-Throughput Isolation and Cultivation

Karsten Zengler

Abstract

The ability to isolate and cultivate cells in the laboratory has been key to progress in the life sciences. Methods that allow for automated and rapid isolation and cultivation of microorganisms in the laboratory can provide access to organisms that have previously not been propagated in the laboratory, thereby enabling in-depth studies of the physiology of these microbes. Here we describe an automated high-throughput method that combines encapsulation of cells into agarose microcapsules. After isolating individual cells by encapsulation, the population of cells can be incubated as a whole. Cells that divide and form distinct microcolonies within the microcapsule can subsequently be separated from each other by flow cytometry.

Keywords: Cultivation, Flow cytometry, High throughput, Isolation, Microcapsules

1 Introduction

The isolation, growth, and cultivation of microorganisms have been paramount to our knowledge and understanding of them. Domesticating microorganisms in the laboratory allowed generations of researchers to study them under controlled conditions. Through these experiments we obtained insight into how information stored in the genome is accessed to generate diverse phenotypes. By studying the genotype to phenotype relationship, scientists realized that there is more to a microorganism than a bunch of genes stitched together. The complex relationship between transcription and translation allows microbes to react and interact with their environment and other organisms in a highly dynamic way. Only through a deep understanding of the mechanistic basis for these interactions will we be able to predict and control microbially mediated processes. Thus, the isolation and cultivation of microbes lays the foundation for all of microbiology and is crucial for almost all

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microbe-related fields of research, from synthetic biology, metabolic engineering and biotechnology, to microbial ecology, medical and applied microbiology, to mention just a few.

Often the terms "isolation," "growth," and "cultivation" are used interchangeably although they refer to three distinct processes on the way to establishing microorganisms in the laboratory. Isolation refers to the physical separation process in which cells are separated from other cells or extracellular matrix, such as host tissue or soil. Growth describes the division of a cell, ultimately resulting in an increase in cell number, while cultivation implies propagation and maintenance of cells in the laboratory. Isolation is most critical for our cultivation efforts but arguably the most disruptive process. Traditionally we tend to propagate bacteria as pure cultures and thus consciously remove all interaction of the cultures with their environment. Successful growth therefore relies on how well we are able to mimic or substitute growth requirements in the laboratory.

Several methods commonly used to physically isolate cells exist. Traditionally, cells are spread on a solid medium surface, e.g., agar, and picked from the surface after a colony or microcolony [1–4] has been established. In this case the isolation takes part after growth. Methods by which cells are being isolated before microbial growth occurs include liquid serial dilution [5–8], flow cytometry [9–11], microfluidics [12–15], or micromanipulation using focused laser beams, so-called optical tweezers [16, 17]. Common to all techniques that isolate microorganisms before growth is their ability to detect and subsequently separate individual cells. An approach that does not require single cell detection for separation is the isolation of bacteria by encapsulation in microcapsules and sorting by flow cytometry [18]. This method, described in detail below, encapsulates individual cells in agarose beads by dispersing low-melting agarose and microbes into an immiscible solvent (i.e., mineral oil), forming an emulsion by blending. Agarose beads containing embedded cells are separated from the mineral oil by multiple centrifugation and washing steps. All cells are subsequently incubated together allowing cells to divide and form microcolonies. Agarose beads that contain microcolonies can then be separated by flow cytometry, resulting in pure cultures.

2 Materials

1. Set two water baths to 80 and 45°C.
2. 1.5% low-melting agarose (SeaPlaque Agarose, Lonza), dissolved in medium or salt solution according to cell's origin (*see* below).
3. Mineral oil can be purchased from laboratory supply vendors. Alternatively, mineral oil available at drug stores (i.e., paraffin

oil, often sold to treat bowel irregularity) can be used as a cost-effective variant.

4. Magnetic microparticles (e.g., beadMAG particles) can be purchased from laboratory supply vendors (ChemiCell, Germany). Cotton color is available in most craft shops. Correct fluorescence emission of these colors can be tested using a fluorescence microscope/FACS machine.
5. 100× Pluronic acid F-68 (Life Technologies).

3 Methods

3.1 Encapsulation

The encapsulation protocol requires two water baths set at 80 and 45°C, respectively.

1. Prepare a 1.5% solution of SeaPlaque Agarose (Lonza) in a 1× PBS. If microorganisms are being encapsulated from environments that contain higher salt concentrations, the PBS can be replaced by a mineral medium containing the appropriate salt concentration. For marine microbes, sterile filtered seawater is best suited; for microbes from oil wells, production water is a good source as medium.
2. Dissolve the agarose by boiling in a microwave oven.
3. Aliquot 1 mL of the agarose solution in 1.5 mL Eppendorf tubes.
4. Add 60 μL Pluronic acid (100× Pluronic F-68, Life Technologies) to the 1 mL of agarose solution, mix briefly on a vortex, and keep dissolved by storing it in the 80°C water bath.
5. Fill scintillation vials with 15 mL mineral oil. Preheat the mineral oil to 45°C.
6. Transfer melted agarose to 45°C, wait 5 min to equilibrate to the right temperature.
7. Prepare cells for encapsulation by diluting cells in the appropriate buffer (same buffer as is used to generate the agarose solution).
8. The cell concentration should be checked by microscopy. A suitable concentration is approximately 10–30 cells per field of view at a 400× magnification.
9. Pipet 200 μL of the diluted cells into the agarose and briefly mix by vortexing. Additionally, 20 μL of magnetic microparticles or 20 μL of cotton color can be added here, allowing for later separation/discrimination of discrete populations (*see* **Note 3**).
10. Pipet 1 mL of the agarose-cell mixture into the 15 mL of preheated mineral oil (Fig. 1a). Up to 2 mL of agarose-cell mixture can be combined in one vial of 15 mL mineral oil.
11. Close the scintillation vial and shake vigorously for 10 s.

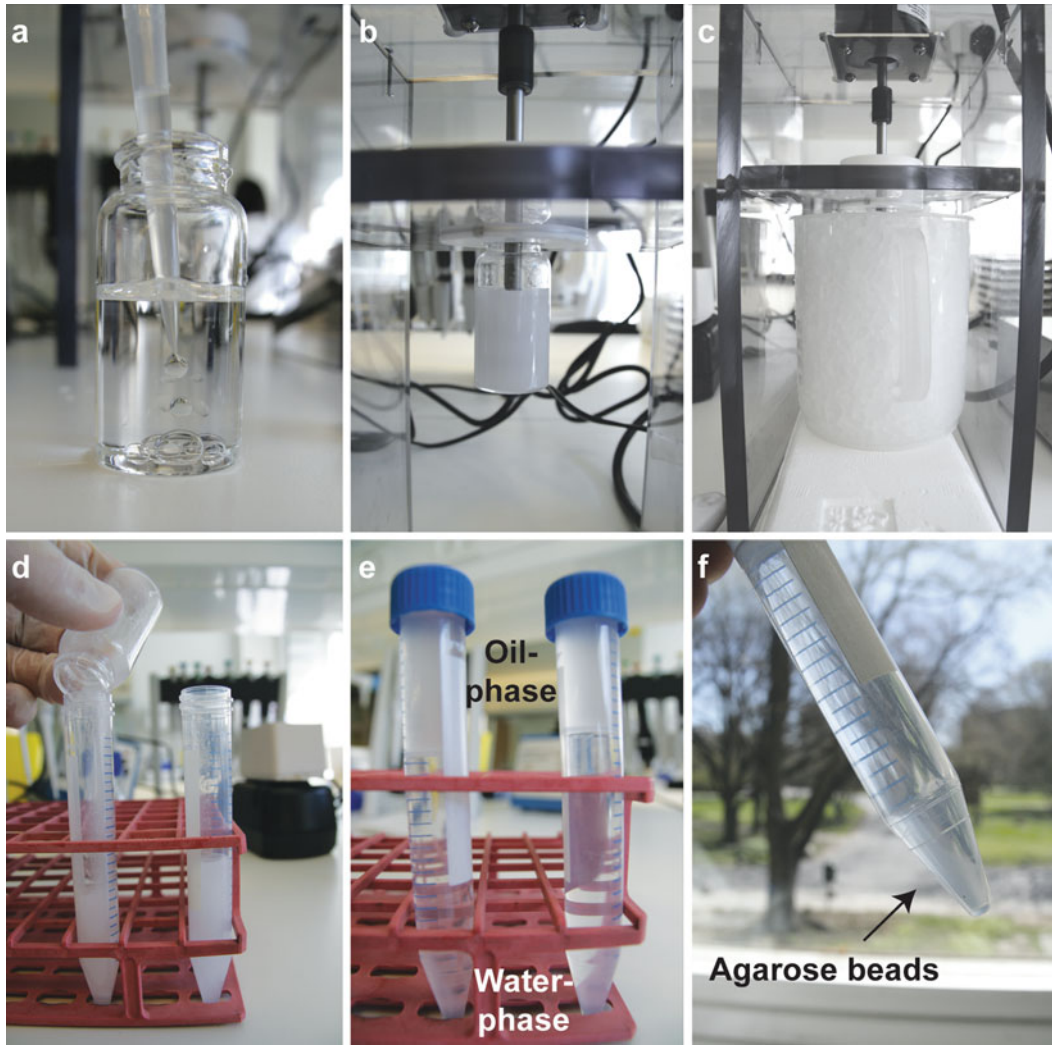

Fig. 1 Encapsulation of cells in agarose beads. (**a**) Agarose and bacteria are mixed at a ratio of 15:1 in a scintillation vial containing preheated (45°C) paraffin oil. (**b**) Oil and agarose are emulsified using a blender to generate microcapsules. (**c**) The emulsion is rapidly cooled down using an ice bath to solidify the microcapsules. (**d**–**f**) Washing steps performed in 15 mL tubes. The oil is separated from the water phase (containing agarose particles) by repeated centrifugation. An agarose pellet (**f**) is formed which, after additional filtration, will be incubated for downstream processes

12. Transfer the vial to the microcapsule maker and screw scintillation vial into holder (Fig. 1b).
13. Blend emulsion (*see* **Note 1**). The following protocol is optimized for a given microcapsule maker (One Cell Systems) to generate microcapsules of 10–50 μm in size. Results with different microcapsule makers will vary based on rotation speed, as well as blade design, and should be optimized prior to encapsulation. Mix the solution at 2,900 rpm for two minutes at room temperature followed by 1 min of mixing at 2,900 rpm

on ice (by placing the vial into an ice bath). Continue mixing on ice at 1,500 rpm for 6 min (Fig. 1c).

14. Transfer the oil/agarose emulsion equally into two 15 mL tubes and add ~10 mL of PBS or appropriate buffer into each tube (Fig. 1d).
15. Centrifuge the tubes at 2,000×*g* for 10 min.
16. Remove all the mineral oil and PBS from the microcapsule pellet and discard in biological hazardous waste container (Fig. 1e).
17. Resuspend the microcapsules in PBS or appropriate buffer and transfer to a new 15 mL tube, combining two tubes into one.
18. Centrifuge again at 2,000×*g* for 5 min and remove supernatant.
19. Resuspend microcapsules in appropriate buffer and transfer to new tube and repeat centrifugation (2,000×*g* for 5 min). Normally these wash steps remove all mineral oil from the microcapsules (Fig. 1f). If mineral oil can still be spotted another centrifugation is required.
20. Resuspend the final microcapsule pellet in 4 mL of appropriate buffer.
21. Check your microcapsules for size, homogeny, and encapsulation efficiency by microscopy. Using 2 mL agarose-cell mixture will result in approximately 10^{14} microcapsules total. There will be a size distribution of microcapsules, but ideally most capsules should be around 20 μm in size. The microcapsules should look smooth under the microscope. Grainy microcapsules will decrease sorting efficiency by flow cytometry.
22. Evaluate the encapsulation efficiency by staining the cells with standard fluorescent stains, such as SYBR Green or LIVE/DEAD stains (Life Technologies), and look for cells inside the microcapsules using a fluorescent microscope. The encapsulation protocol will result in approximately 10% of microcapsules containing ideally a single cell. The number of cells per microcapsule can be varied by adding different amounts of cell solution to the agarose mixture at the start of the procedure (Step 9).
23. If large agarose particles and clumps are observed under the microscope, filter the microcapsules using custom made 30 μm nylon-mesh filter mounted in Pop-top type syringe filter holders (Whatman). Alternatively, cheesecloth can be used to filter agarose particles prior to incubation.

3.2 Incubation

The encapsulated cells in microcapsules will be incubated ideally in a flow-through system. The idea behind this is that nutrients, e.g., from oil production waters, can be provided at low concentrations over an extended period of time. We often used flow rates of >10 mL/h to achieve this. The constant flow also has the

advantage that free cells will be diluted or washed out of the system (*see* **Note 2**). Several systems can be deployed for this flow-through incubation setup. We had good experience with preparative liquid chromatography glass columns, which were incubated in an upright position keeping the microcapsules toward the bottom of the column. The medium inlet was also at the bottom of the column. Depending on the flow rate of the medium, the microcapsules can be kept in a hovering position over the inlet, allowing for optimal flow of nutrients. This setup enables the user to remove most free cells that originate by bursting microcapsules of faster-growing cells while maintaining capsules with slow-growing organisms in the system.

3.3 Sorting

After the microcapsules have been incubated for an appropriate amount of time, microcolonies will develop inside the microcapsules. Since microcolony development is species dependent, best results are obtained when capsules are sorted at different time points.

1. Harvest up to 15 mL of column contents by pipetting up and down. The microcapsules start sinking to the bottom of the column after prolonged incubation times.
2. Centrifuge microcapsules at 2,500×*g* for 5 min.
3. Discard supernatant into biological hazardous waste container and resuspend pellet in 10 mL appropriate buffer.
4. Wash microcapsules by repeated centrifugation (2,500×*g*, 5 min) and resuspend in ~4 mL buffer.
5. Filter suspended microcapsules through a 40 μm filter to remove large microcapsules that could clog the flow cytometer.
6. Operate the flow cytometer according to the manufacturer's instruction using an appropriate nozzle size (80 μm or larger is recommended).
7. Sort microcapsules individually or as pairs of multiple capsules (depending on the research question) into the desired incubation vessel such as microtiter plates, tubes, agar plates, etc.

4 Notes

Note 1

1. Special attention should be given to the encapsulation process. The size and quality of the microcapsules are critical for all downstream processes. The size largely depends on the microcapsule maker, the form and size of its blade, and the blending speed. Different equipment will thus result in different microcapsule sizes. The size of the microcapsules follows a standard

distribution curve. While large microcapsules can be easily removed by filtration, small capsules will cause a problem by increasing overgrowth of the microcapsules by free cells during incubation (*see* **Note 2**).

2. It is recommended that microcapsule generation is optimized for the equipment and type of agarose used prior to encapsulation. The quality of the microcapsules depends on the blending speed but also on the agarose used. Suboptimal formation of the microcapsules can result in grainy microcapsules, which will influence the downstream sorting.
3. Microcapsules that themselves produce a signal in the flow cytometer (forward/side scatter) should be avoided. The size distribution as well as the graininess should therefore be evaluated by microscopy.
4. It could be beneficial for certain experiments (e.g., increased incubation temperature) to use a different polymer for encapsulation. If different polymers or mixtures of polymers are used, blending and mixing needs to be adapted and optimized.

Note 2

1. Incubation of cells encapsulated in microcapsules will result in growth of the cells and formation of microcolonies within the microcapsules. Depending on the location of the microcolony within the microcapsule and how fast the cells grow, a release of the cells from the capsule can occur. Cells that are closer to the outside of the microcapsule will form colonies that will grow out of the microcapsules faster than cells encapsulated at the center of the microcapsule. This will produce free cells in the incubation vessel. Free cells can cause several negative effects on the experimental outcome. The cells can attach to other microcapsules and thus produce mixed cultures after sorting. We also observed that these cells swim or grow as biofilms toward the medium source.
2. Use two 0.2 μm filters in a row to prevent cells moving into the medium reservoir.
3. Adjust the flow rate of the medium into the incubation column to reduce the amount of free cells to a minimum. This flow rate depends on the growth rate of the cells and the medium concentration used for the incubation.
4. In case incubation is not performed in a flow-through system but in a different vessel (such as an Erlenmeyer flask), microspheres can be augmented with magnetic microparticles (Step 9 during encapsulation) during the encapsulation process (Fig. 2). This enables the subsequent separation of microspheres from planktonic bacteria using a magnetic Eppendorf rack and repeated washing steps.

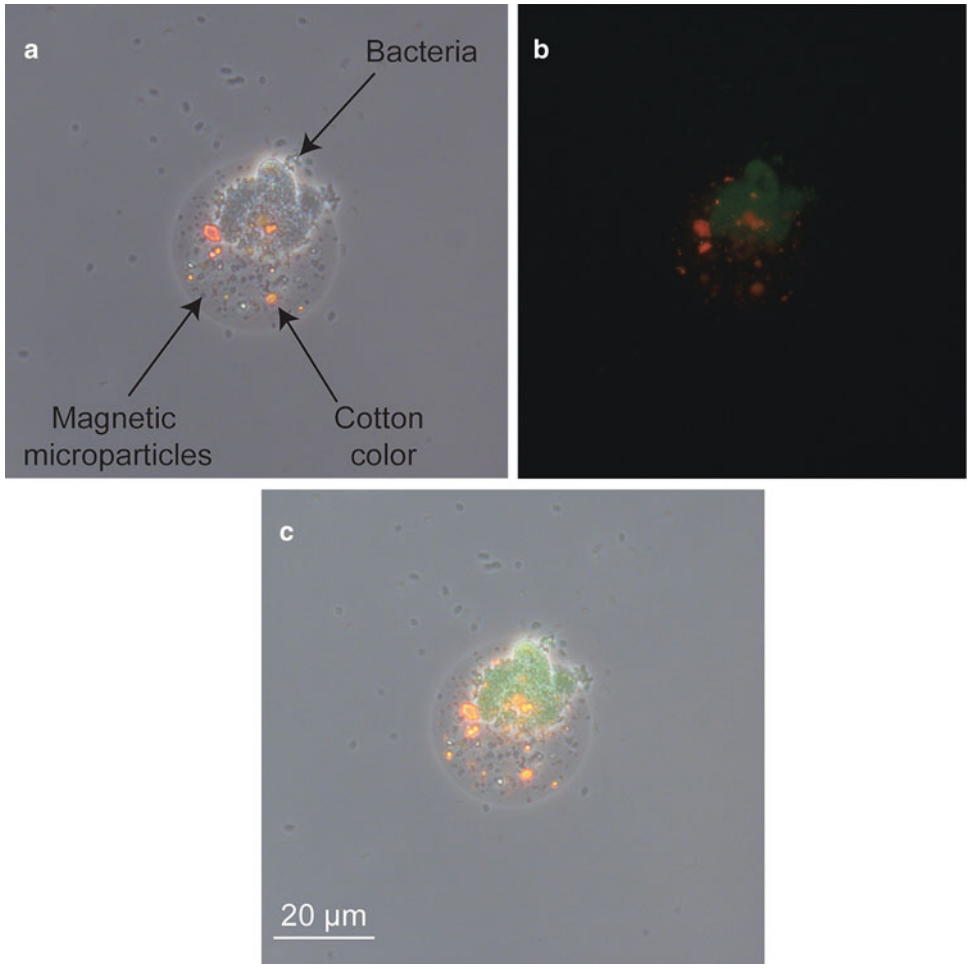

Fig. 2 Microscopic images of encapsulated bacterial cells. (**a**) Brightfield images of an agarose microcapsule containing *E. coli* cells expressing GFP, *red cotton color*, and magnetic microparticles. The *red cotton color* is clearly visible as large red particles within the beads and can be used to discriminate populations by microscopy or flow cytometer. Embedded magnetic microparticles are used to magnetically separate agarose microcapsules from planktonic cells, thereby reducing the background noise during flow cytometry. (**b**) Fluorescence image (ex. 450–490 nm, em. >510 nm) of stained microcapsules (*red cotton color*) containing *E. coli* cells expressing GFP and red cotton color. (**c**) Combined brightfield and fluorescence image

Note 3

1. Staining of microcapsules using standard cotton dye colors can be applied to separate populations based on their specific color (Fig. 2). This can be a convenient tool to guide separation of different populations based on the respective fluorescence emission.

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Single Bacteria Studies Using Microfluidics

Yanqing Song, Bing Li, Yong Qiu, and Huabing Yin

Abstract

The importance of individual heterogeneity within a genetically identical population has become well recognized. However, single bacteria studies have been beset by a number of challenges ranging from single-cell handling to detection. Most of these stem from bacteria's microscale dimensions and the complexity of their natural environment. In recent years, microfluidics has emerged as a powerful tool to manipulate single cells and their immediate microenvironments and is well suited to address these challenges. The protocols below will describe the creation of microfluidic devices for monolayer cell culture and long-term tracking of morphological dynamics from individual bacteria under precisely delivered perturbations. Step-by-step procedures for on-chip assays and morphological-based image analysis are described in detail, and these approaches enable fast quantification of bacteria growth and morphological changes under a broad range of conditions within a single experiment. Importantly, these methods do not require labeling of cells, thereby offering unique advantages in the investigation of naturally occurring microbes.

Keywords: Antibiotics, Chemostatic culture, Concentration gradient, Growth rate, Microfluidics, Morphological tracking

1 Introduction

The importance of individual heterogeneity in determining bacteria fate has become well recognized in many circumstances, such as antibiotic resistance [1, 2] and function of bacterial communities [3, 4]. To reveal such heterogeneity in a population, statistically meaningful data from a substantial number of individual cells are required. However, conventional well-plate-based assays measure the average response from a population of cells and have limited capability to control local environments surrounding individual cells.

Single-cell studies have been beset by a number of challenges. Firstly, single-cell handling is difficult because of their small dimensions (~1 μm) and motility. Secondly, naturally occurring microbes live in complex communities, and therefore, isolated cells may lose certain functions due to the loss of their natural habitat. Furthermore, current methods for characterization of bacterial

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species and phenotypic function are often restricted by the availability of specific probes (e.g., immunofluorescence probes for microorganisms [5]).

In recent years, microfluidics has emerged as a powerful enabling technology for single-cell analysis, leading to a plethora of unprecedented discoveries [6–9]. Typical microfluidic channels are in the order of tens of microns. At this length scale, laminar flow dominates, which permits the accurate control of cells and molecule trajectories within the channel through a variety of means (e.g., microchannel geometries and electrical fields) [10–12]. Furthermore, microfluidics can precisely control thousands of ultralow sample volumes (as low as pico-liter), thus enabling massively parallel analysis of low numbers of samples [13–15]. A recent example of such powerful capability is the single-cell genomic analysis chip where isolation and genome amplification of individual microbial cells has been accomplished on the same chip [16, 17]. In recent years, numerous insights have been gained as a result of the exploitation of microfluidic technologies in microbiology [6, 18].

Here, we will highlight the recent development of microfluidic technologies for long-term monitoring of bacteria growth at the single-cell level. Bacterial growth is associated with a myriad of fundamental biological phenomena, such as aging, antibiotic resistance, and quorum sensing [7, 19, 20]. Quantitative measurement of growth rates for single cells under well-controlled perturbations is key to understanding these phenomena. With microfluidic technologies, experiments are conducted on the small scale, enabling precise control of the environments around individual cells and high-resolution imaging of cells (e.g., time-lapse imaging). Among the microfluidic devices developed so far, a common feature is to trap cells so that imaging of individual cells is possible. Different means have been developed, resulting in 3D multilayer [21, 22], 2D monolayer [23], and 1D linear track [24, 25] growth of cells. It should be noted that controlling microflows has been explored in all of these cases so as to deliver the desired conditions, for instance, the creation of nanoliter-scale batch culture [26], chemostatic culture [27–29], and programmable delivery of culture medium [25].

In this protocol, we will provide details to create microfluidic devices for monolayer cell culture and long-term tracking of morphological dynamics from individual bacteria under precisely delivered perturbations. Based on morphological changes, the growth rates of individual cells are quantified, and single-cell response to the defined stimuli is revealed. These approaches have provided a fast and reliable means for both quantitative and mechanistic investigations of bacterial inhibitors [30] and have led to the discovery of a new form of bacterial persistence [31].

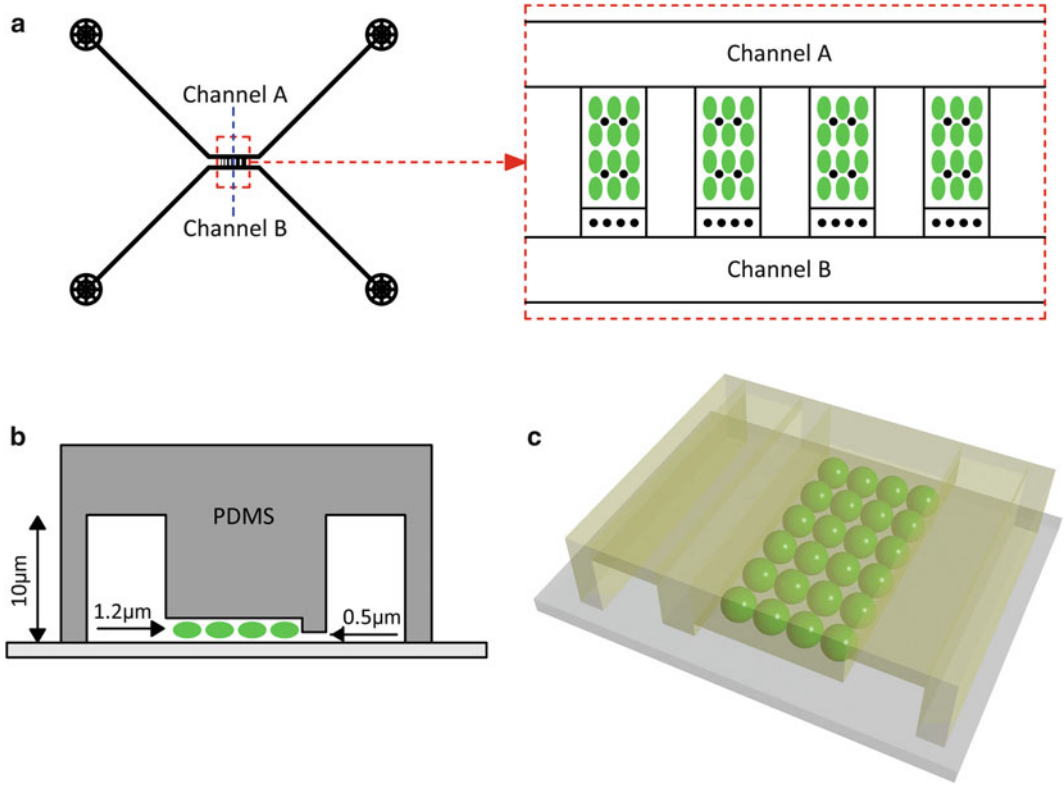

Fig. 1 Schematic drawing of the enclosed terrace microfluidic device. (**a**) Top view of the device and the three layers. *Green balls* represent cells and black dots represent micropillars within the chambers. (**b**) Cross-sectional view of the device. The barrier channel is 0.5 μm high. The trapping chamber, where a monolayer of cells are trapped, is 1.2 μm high. The two flow channels are 10 μm high. (**c**) 3D illustration of a part of the device. Green spheres represent trapped cells

Considering the accessibility of microfabrication facilities (e.g., clean room), two configurations of microfluidic devices for monolayer cell culture will be described below:

1. An enclosed terraced microfluidic device that requires access to microfabrication facilities (Fig. 1), but, after fabrication, is ready to use immediately and offers a high-degree control over microflow.
2. A microfluidic device assembly that requires manual skills to assemble, but can be readily implemented in conventional microbiology labs (Fig. 2) [30, 31].

Both devices contain two parallel microchannels to deliver medium and perturbations over the monolayer of cells. For the purpose of clarity, we will describe two of the procedures used in detail, namely, (1) the exchange of solutions and (2) the formation of concentration gradients. The advantages and limitations of the two devices for these operations will be discussed. It is envisaged that the platforms can be easily adapted for a variety of applications.

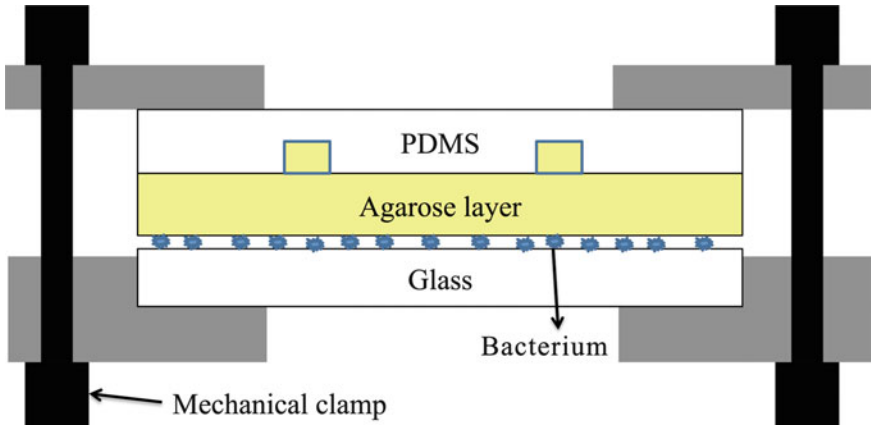

Fig. 2 Schematic drawing of the manually assembled microfluidic device (cross-sectional view)

It should be noted, although only *E. coli* is described here, the protocol is applicable to any bacterium, such as *Nitrosomonas europaea* – an ammonia-oxidizing bacterium.

2 Materials

2.1 Silicon Mold Fabrication

- Photolithography mask layout software
- Silicon wafers (4 inch, University Wafer)
- Positive photoresist, e.g., Microposit S1818 (Rohm and Haas)
- S1818 developer (Microposit MF™-319, Rohm and Haas)
- Cross-linked negative photoresists, e.g., SU-8 2000.5, SU-8 2002, and SU-8 3010 (MicroChem)
- SU-8 developer (Micro EC developer, MicroChem)
- Metal evaporator (Plassys MEB 550S Electron Beam Evaporator)
- Spin coater (Headway PWM32 spinner)
- Mask aligner (Süss MicroTec MA6 mask aligner)
- Chemical reagents: acetone, methanol, isopropyl alcohol, and trichloro(1H,1H,2H,2H-perfluorooctyl)silane. All the reagents are from Sigma.

2.2 Formation of the Two Microfluidic Devices

- Polydimethylsiloxane (PDMS) base agent and curing agent (Sylgard 184 Silicone Elastomer Kit; Dow Corning)
- Vacuum desiccator (Cole-Parmer)
- Scalpels (stainless steel blade with handle)
- Biopsy hole punches (tip inner diameter = 0.5 mm, World Precision Instruments. Cat. No. 504528)

2.2.1 Preparation of the PDMS Chip Used to Introduce Solutions and Media

2.2.2 The Enclosed Terrace Device

- PDMS chip prepared in Sect. 2.2.1
- Coverslips (22 × 40 mm, Fisher, Cat. No. 12352128)
- Oxygen plasma asher (GaLa Instrumente, Plasma *Preps*)

2.2.3 The Manually Assembled Microfluidic Device

- PDMS chip prepared in Sect. 2.2.1.
- A mechanical clamp made in-house that consists of top and bottom plexiglass plates to hold an assembly of microfluidic devices (Fig. 2).
- Agarose: low gelling temperature agarose (Sigma, Cat. No. A9045).
- Coverslip (24 × 24 mm, Fisher, Cat. No.12302118). The coverslip should fit into the recess in the bottom plexiglass plate.
- PDMS spacer with uniform thickness of 0.25 mm made in-house (*see* Sect. 3.2.3).

2.3 Preparation of Bacteria Cells

- Strains: such as *E. coli* strain (ATCC, Cat. No. ATCC 25922).
- Luria-Bertani (LB) broth: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, sterilized (20 min at 121°C). All chemicals are of analytical grade purity.
- Buffer: phosphate buffer saline (PBS) (Sigma, Cat. No. 319252).
- Biological safety hood (ESCO Class II Biohazard Safety Cabinet).

2.4 On-Chip Assays

- Cell solutions prepared as described in Sect. 2.3
- Two microfluidic devices prepared as described in Sect. 2.2
- Syringe pumps (NE-4000, New Era Pump Systems Inc., http://www.syringepump.com)
- Tygon tubing (Cole-Parmer, Cat. No. EW-06419-01)
- Blunt needles (23G × 1″) (Intertronics, Cat. No. FIS5601101) (*see* **Note 1**)
- Silicon adhesive (Dow Corning, 3145 RTV MIL-A-46146)
- Plastic syringe: 1 and 5 mL (BD, http://www.bd.com)
- 4-port valves (Cole-Parmer, Cat. No. TW-06471-70)
- Distribution valves (5 ports, one port to inlet) (Cole-Parmer, Cat. No. TW-06471-72)
- Screw-in valve (Cole-Parmer, Cat. No. TW-06473-22)
- Amoxicillin (Sigma, Cat. No. A8523)
- Fluorescein: fluorescein isothiocyanate isomer I (Sigma, Cat. No. F4274). Prepared as a 30 mM solution in LB broth and stored at 4°C

2.5 Time-Lapse Imaging Acquisition

- Automatic inverted fluorescence microscope (Zeiss AxioObserver Z1)
- Objectives (40×, NA 0.75, plan objective, Zeiss)
- CCD camera (Photometrics Cascade II)
- AxioVision4 software (Zeiss)

2.6 Imaging Analysis

- Image J software (Ver 1.48 m, http://rsb.info.nih.gov/ij/download.html)

3 Methods

The protocols below describe two configurations of microfluidic device for single-cell analysis under well-controlled microenvironments, namely, an enclosed terrace chip and a manually assembled device. Both enable chemostatic, monolayer culture of bacteria and long-term tracking of single-cell morphological changes. The enclosed terrace chip requires advanced microfabrication, but offers a number of application advantages involving fast delivery of perturbations to the media composition, compatibility with studies of growth generations, and a high degree of flexibility in creating flow patterns around the bacteria cells. The main advantage of the manually assembled device however is that it can be readily implemented in a conventional microbiology lab without the need of specialized instruments and is well suited to establish long-term, stable concentration gradients.

3.1 The Silicon Mold for PDMS Chip Fabrication

Silicon molds serve as the masters for making the PDMS replica that either form the bacterial culture device itself or are the interface required to transfer solutions to the cultures (*see* Sect. 3.2). In the case of the terrace chip, a three-layer silicon mold is required in which the layers have precisely controlled thicknesses, namely, layer one, 0.5 μm thick; layer two, 1.2 μm thick; and layer three, 10 μm thick (as shown in Fig. 1). The smallest dimension of a "typical" bacterial cell is about 1 μm. Therefore, with this terraced device, they can be confined as a monolayer of cells in the middle layer of this structure. Considering that the silicon mold for the manually assembled device is easy to fabricate, only the procedure for fabricating the terrace device mold is described below (and shown in Fig. 3).

3.1.1 Design of Devices

1. The device is designed using layout software such as L-edit. For the terrace device, masks for the three layers need to be designed separately. Crosses, acting as alignment marks, must be included in each layer (as shown in Fig. 3a).

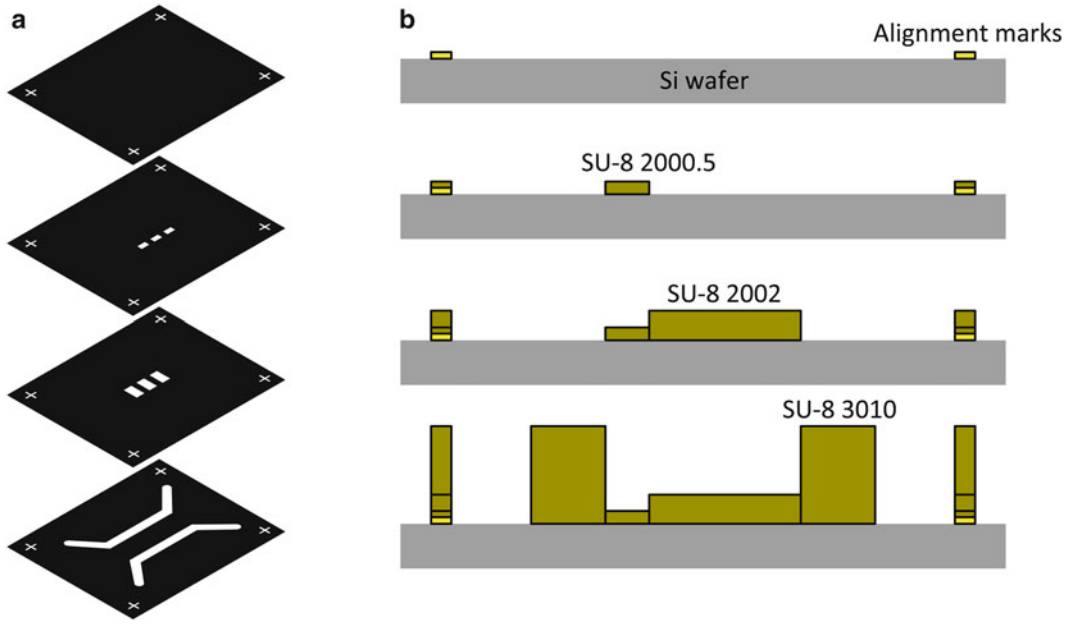

Fig. 3 Outline for silicon mold fabrication. (**a**) Mask design for multilayered silicon mold. Firstly, a mask is needed to create alignment marks. Then three separate masks are needed for photolithography. (**b**) Fabrication process. S1818 and nichrome are used to produce alignment marks. SU-8 photoresists are spin-coated on a silicon wafer, exposed to UV radiation, and developed. The thickness of each SU-8 layer determines each channel's height

2. Chrome photomasks are produced based on the L-edit designs (in our instance, this production was done in-house: www.jwnc.gla.ac.uk).

3.1.2 Fabrication of Alignment Marks

1. Clean the silicon wafer in acetone, methanol, and isopropyl alcohol sequentially in an ultrasonic bath for 5 min, followed by blow drying with nitrogen gas.
2. Dehydrate the wafer in an oven at 180°C for 10 min.
3. Spin-coat S1818 on the wafer at 4,000 rpm for 30 s, and soft-bake the wafer on a hot plate at 110°C for 3 min.
4. Load the photomask with alignment marks only, together with the S1818-coated wafer into the mask aligner. Expose the wafer to UV light at a dose of 100 mJ/cm^2.
5. Develop the wafer in MF-319 developer for 75 s. Rinse the wafer with deionized water and dry it with nitrogen gas.
6. Place the wafer in the plasma chamber and treat with oxygen plasma for 5 min at 200 W to descum any residual resist.
7. Deposit 30 nm NiCr on the wafer using a metal evaporator.

8. Immerse the wafer in warm (e.g., 50°C) acetone until all photoresist is lifted off. Dry the wafer with nitrogen gas. This results in the wafer with alignment marks (as shown in Fig. 3b).

3.1.3 Fabrication of Three-Layer Silicon Mold

1. Dehydrate the wafer with alignment marks in an oven at 180°C for 10 min.
2. Spin-coat SU-8 2000.5 on the wafer at 3,000 rpm for 30 s to fabricate the first layer, and soft-bake the wafer on a hot plate at 95°C for 1 min.
3. Align the photomask for the first layer and the wafer on the mask aligner. Expose to UV radiation at a dose of 60 mJ/cm^2.
4. Postbake the exposed wafer on a hot plate at 95°C for 1 min.
5. Immerse the wafer in SU-8 developer for 10 s (*see* **Note 2**).
6. Rinse the wafer with isopropyl alcohol and blow dry with nitrogen gas.
7. Hard-bake the wafer in an oven at 180°C for 10 min.
8. Spin-coat SU-8 2002 on top of the wafer at 5,000 rpm for 30 s to fabricate the second layer, and soft-bake the wafer at 95°C for 1 min.
9. Align the photomask for the second layer and the wafer on the mask aligner. Expose the wafer to UV light at a dose of 80 mJ/cm^2.
10. Postbake the exposed wafer on a hot plate at 95°C for 2 min.
11. Develop the wafer in SU-8 developer by immersion for 15 s. Rinse the wafer with isopropyl alcohol and blow dry with nitrogen gas.
12. Hard-bake the wafer in an oven at 180°C for 10 min.
13. Spin-coat SU-8 3010 on top of the wafer at 3,000 rpm for 30 s to fabricate the third layer, and soft-bake the wafer at 95°C for 3 min.
14. Align the photomask for the third layer and the wafer on the mask aligner. Expose the wafer to UV light at 200 mJ/cm^2.
15. Postbake the wafer on a hot plate at 65°C for 1 min, followed by baking at 95°C for 2 min.
16. Develop the wafer in SU-8 developer by immersion and agitation for 1 min (*see* **Note 2**). Rinse the wafer with isopropyl alcohol and blow dry with nitrogen gas.
17. Hard-bake the wafer in an oven at 180°C for 20 min.
18. Put the wafer in a vacuum desiccator with a glass slide placed next to the wafer. Drop ~20 μL trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane on the glass slide, and evacuate the desiccator to expose the wafer to the silane vapor overnight. The silanized silicon mold can be used many times for PDMS replica molding (*see* **Note 3**).

3.2 Formation of the Enclosed Terraced or Manually Assembled Microfluidic Devices

PDMS replica molding enables production of a large number of microfluidic devices at low cost, making them disposable. For both microfluidic devices, the PDMS replica chip is the main component and thus its production will be detailed first in Sect. 3.2.1. This is followed by the description for the formation of the two devices (i.e., Sects. 3.2.2 and 3.2.3).

3.2.1 Preparation of PDMS Chip

1. Weigh the liquid of PDMS curing agent and base agent (oligomer) in a ratio of 1/10 (w/w), and stir thoroughly.
2. Fix the silanized silicon mold prepared in Sect. 3.1 to the base of a petri dish using double-sided tape, and pour the PDMS liquid mixture on top of it to produce desired thickness of chip (~5 mm).
3. Place the petri dish under vacuum for 30 min to remove bubbles.
4. Place the petri dish in an oven at 75°C for 3 h.
5. Cut the cured PDMS replica along the edge of the mold by using a scalpel, and detach it off the silicon mold carefully.
6. Punch 0.5 mm holes in the PDMS slab as inlets and outlets.

3.2.2 Making the Enclosed Terrace Device

The enclosed terrace chip consists of a PDMS chip irreversibly bonded to a coverslip. Note, several devices can be manufactured at the same time and stored for several months at room temperature, before use.

1. Clean the PDMS chip and a glass coverslip with acetone, methanol, and deionized water, followed by blow drying with nitrogen gas.
2. Activate the glass coverslip and the channel side of the PDMS chip in the oxygen plasma chamber at 100 W for 30 s.
3. Immediately bring the exposed surfaces together. Place the device in an oven at 65°C for 5–10 min (*see* **Note 4**).
4. Store the devices in a clean container.

An example of the enclosed terrace device is shown in Fig. 4.

3.2.3 Making the Assembled Microfluidic Device

The assembly device consists of several components, namely, a PDMS chip, agarose membrane, PDMS spacer, and a coverslip (as shown in Fig. 5a). The device is best prepared immediately prior to experiments since cell loading is completed during the assembly of the device (*see* Sect. 3.4.2). Here, preparation for the assembly (including the fabrication of PDMS spacers) is described.

1. Prepare a 1/10 (w/w) mixture of curing agent/base agent of PDMS, and stir thoroughly.

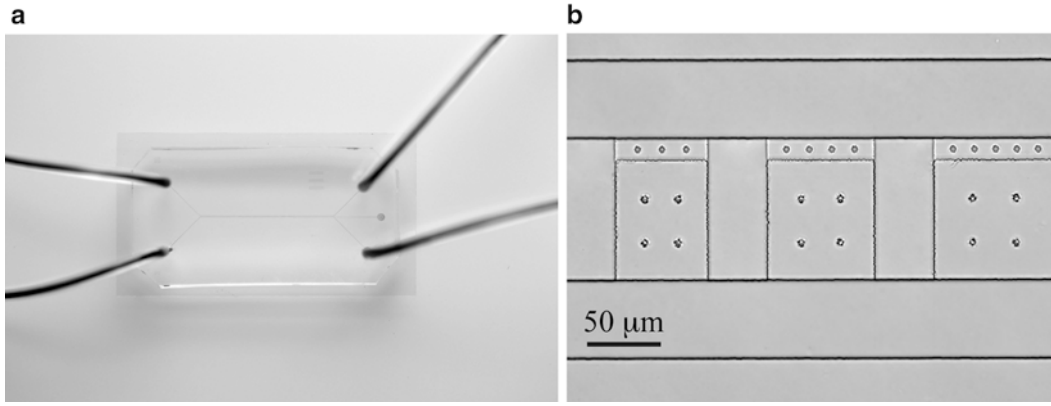

Fig. 4 Optical images of an enclosed microfluidic device. (**a**) Photo of a device with connected tubes. (**b**) Bright-field image of a microchamber region of the chip

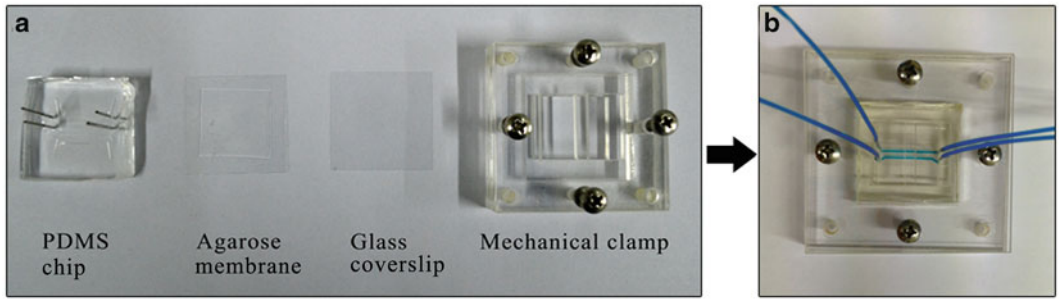

Fig. 5 Photos of an assembled microfluidic device. (**a**) The individual components. (**b**) The final assembly of the device

2. Place a silanized silicon wafer on the spin coater, and drop 5 g of the PDMS mixture in the center of it. Spread the mixture over the whole wafer and spin the wafer at 380 rpm for 1 min. This results in a PDMS film with the thickness of 250 μm.
3. Place the coated silicon wafer in an oven at 75°C for 1 h to cure the PDMS.
4. Remove the formed PDMS film from the silicon wafer, place it on a clean glass microscope slide, and cut it into several hollow squares with an outer dimension of 24 × 24 mm (i.e., the same size as the coverslip) and an inner dimension of 22 × 22 mm.
5. Store the PDMS spacers in a clean container at room temperature.
6. In parallel, prepare a 2% (w/w) agarose solution in deionized water at 65°C in a water bath. The solution needs to be autoclaved and then stored at 4°C.

3.3 Preparation of Bacteria Cells for On-Chip Assays

1. Culture *E. coli* in LB broth in a shaker at 150 rpm at 37°C for around 4 hr to reach its mid log phase. Use OD_{600} value (optical density at 600 nm) to represent the cell mass concentration (this is equal to the absorbance at 600 nm). For *E. coli* this is a certain OD as previously determined. For other cells this will be different.
2. When the OD_{600} value reaches the range 0.6–0.8, harvest the cells. Spin down the bacteria in a centrifuge at 2,400 g for 1 min, discard the supernatant, and wash the bacteria using PBS buffer. Repeat the procedure three times and finally resuspend the bacteria in a fresh LB broth.
3. Dilute the bacterial suspension in LB broth to an OD_{600} value in the range 0.05–0.08.

3.4 On-Chip Assays

Cell loading and controlling of local medium around cells are indispensable processes for on-chip assay. For the assembled microfluidic device, cells are loaded during its construction. However, for the enclosed terrace chip, cells are delivered via solution flow through the inlet made in the PDMS chip. Each approach has its own unique advantages and can be exploited for different applications (*see* **Note 5**). Both devices employ two parallel channels for delivery of solutions, and thus, the flow and solution composition can be controlled independently. Modulation of cell microenvironments via fast solution exchange and the formation of concentration gradients will be described in this section.

3.4.1 With the Enclosed Terrace Device

For the enclosed terrace device, forced flow profiles dominate the exchange of solutions. Therefore, it is well suited for applications requiring fast exchange of medium.

Sterilization of Components

1. Insert metal needles in the outlets/inlets (*see* **Note 6**).
2. Place the device, Tygon tubing, screw-in valve, and distribution valve in a petri dish, and sterilize with a UV-ozone cleaner for 30 min.
3. Cover the petri dish and immediately transfer it to the biological safety cabinet.

Cell Loading and On-Chip Culture

1. In the biological safety cabinet, connect the inlet of Channel A to a syringe loaded with sterile PBS buffer and its outlet to the screw-in valve.
2. Place the device on the microscope stage and the syringe on a pump.
3. Flush the device with PBS buffer at a flow rate of 10 μL/min until the entire device is filled with buffer without any trapped air bubbles (*see* **Note 7**).

4. In the biological safety cabinet, connect the 4-port valve with a syringe loaded with the cell solution (prepared in Sect. 3.3 **step 2**, $OD_{600} = 0.6–0.8$), a syringe loaded with medium (denoted as "medium A"), and a free end of the tubing at the inlet port. Fill another syringe with medium (denoted as "medium B"). Ensure there are no trapped bubbles in the syringes and associated tubings.
5. Disconnect the PBS buffer tubing from the inlet of Channel A, and immediately connect the free end tubing of the 4-port valve. Use two pumps (denoted as pump-A1 and pump-A2) to deliver the cell solution and the "medium A," respectively.
6. Connect "medium B" syringe to the inlet of Channel B. Use another pump (denoted as pump-B) to deliver "medium B."
7. Adjust the 4-port valve to connect the cell port to the inlet port. Deliver cell solution and "medium B" at 5 μL/min until cells flow into Channel A, and then reduce the flow rate to 0.02 μL/min.
8. Close the screw-in valve on the outlet tubing of Channel A and turn off pump-B. This directs flow from Channel A to Channel B, thus trapping bacterial cells in the shallow chambers (*see* **Note 8**).
9. Once cell density in the trapping chamber is satisfied, open the outlet valve of Channel A.
10. Adjust the 4-port valve to connect "medium A" port with the inlet port. Deliver "medium A" and "medium B" at the same speed (e.g., 5 μL/min) to wash away excess cells in the flow channels.
11. For chemostatic culture of cells, maintain both medium flows at a rate of 0.5 μL/min to remove bacterial offspring from the chambers (*see* **Note 9**).

Solution Exchange and Formation of Concentration Gradients

1. For rapid replacement of solutions surrounding cells, forced solution flow is advantageous and therefore should be employed across the cell chambers. This can be achieved using a similar procedure as that for trapping cells (i.e., **steps 7, 8**, and **11**). Instead of a 4-port valve, a distribution valve is recommended to avoid adjusting the valve for different solutions. Therefore, the delivery of each solution can be controlled by programming the pumps (*see* **Note 10**).
2. By replacing either of the regular medium in the two channels with a medium containing a high concentration of a compound, a concentration gradient of the compound across the two channels can be formed. However, small changes in flow rate (e.g., syringe pump pulsing) can cause this gradient to

fluctuate during long-term measurements, and it is therefore not recommended (*see* **Note 11**).

3.4.2 With the Assembled Microfluidic Device

The use of an agarose membrane in the microfluidic device assembly avoids direct exposure of cells to the flow. As such, the movement of molecules between the two channels and across the cells is driven purely by diffusion, a process that is conducive to the formation of a stable gradient concentration.

Sterilization of Components

1. Clean all the components, namely, the PDMS chip, PDMS spacer, and coverslip with methanol and then 70% ethanol for 5 min each in an ultrasonic bath, followed by blow drying with nitrogen gas.
2. Transfer all the components to a biological safety cabinet, and further sterilize them under UV light for at least 30 min.
3. The sterilized components are assembled immediately for cell loading in the biological safety cabinet, as described below.

Cell Loading and Culture

1. Prepare the sterilized 2% agarose solution at 65°C prior to cell loading, as described in Sect. 3.2.3.
2. Place the PDMS spacer on the coverslip, drop about 0.1 mL of the melted agarose solution in the inner area of the spacer, and immediately cover it with a coverslip to squeeze out excess solution. After 20 min at room temperature, a porous agarose membrane forms.
3. Remove the top coverslip and the PDMS spacer, place the PDMS chip (the side with channels) on top of the agarose membrane, and then remove the bottom coverslip from the agarose membrane.
4. Place a new PDMS spacer around the agarose membrane to reduce the evaporation in agarose gel.
5. Dispense 3 μL of a bacterial suspension (OD_{600} in the range of 0.05–0.08) onto the center of the agarose membrane (*see* **Note 12**). Place a coverslip on the agarose membrane immediately, by contacting one edge of the membrane at first and then placing it down. This leads to spontaneous and uniform spreading of the bacterial suspension over the membrane.
6. Hold the PDMS chip, agarose membrane, and coverslip together and place them into the bottom part of the plastic holder.
7. Place the top plate of the plastic holder and secure the whole assembly using the screws.
8. Insert needles into the inlets/outlets on the PDMS chip, and adjust the tightness of the assembly by tightening or loosening the screws (*see* **Notes 7** and **13**).

9. Load regular LB medium into a 5 mL syringe and LB medium with compounds of interest (e.g., amoxicillin) in another 5 mL syringe. Ensure there are no trapped air bubbles within the syringe and associated tubing.
10. Take the assembly device out of the biological safety cabinet and place it on a microscope stage.
11. Connect the two syringes to the inlets of the source and sink channels.
12. For chemostatic culture of cells, regular LB medium is delivered in both channels at a flow rate of 5 μL/min to provide culturing nutrients as well as remove metabolites produced by the monolayer of cells underneath the agarose membrane (*see* **Note 14**).

An example of the assembled microfluidic device is shown in Fig. 5.

Solution Exchange and Formation of Stable Concentration Gradients

Diffusion is a slow process, which is governed by Fick's law. Taking fluorescein as an example and using the one dimensional diffusion equation ($t = L^2/2D$, where L is the diffusion distance within time t, D is the diffusion coefficient), it will take about 14 min for most of the substances to diffuse 1 mm – the distance between the two channels. Therefore, when exchanging solutions, it is important to allow enough time for the system to reach an equilibrated state. However, this method has been found to be highly suited to establishing long-term, stable concentration gradients. These enable quantitative evaluation of a wide range of concentrations on the same chip, thus providing rich data for a rapid assay. The procedure for time-lapse tracking of *E. coli* under a stable gradient of amoxicillin is detailed below.

1. Place a syringe loaded with medium containing a high concentration of amoxicillin (e.g., 5 mg/L) and a syringe loaded with fresh medium on a pump. The associated channels are denoted as the source and the sink.
2. Start the delivery of solutions at a speed of 5 μL/min and time-lapse recording. After 20 min, the concentration gradient of fluorescein (or amoxicillin) reaches steady state (as shown in Fig. 6a, b); this can be maintained for days (*see* **Notes 15** and **16**).
3. After the completion of the on-chip assays, replace the syringe containing amoxicillin with a syringe containing 10 mM fluorescein solution. Repeat **step 2** and **3** for 1 h to acquire the gradient calibration curve by fluorescence (*see* **Note 17**).

3.5 Time-Lapse Image Acquisition over a Large Area

The procedure is identical for all the on-chip assays.

- Optimize the microscope setting for bright-field imaging with an appropriate objective lens (e.g., 40×, NA 0.75, Zeiss). Ensure the image background is as clear as possible.

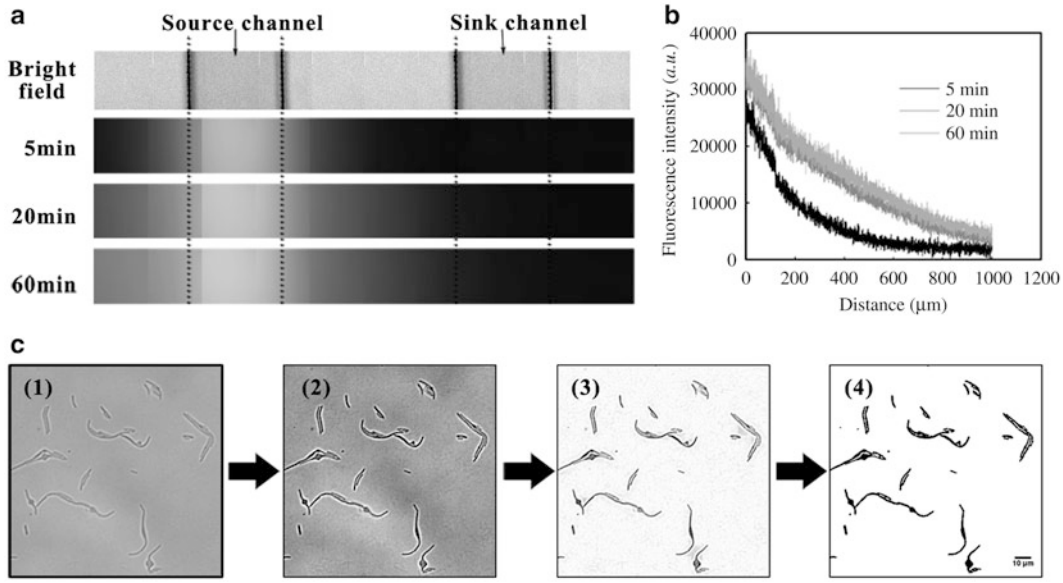

Fig. 6 Concentration gradient formed under agarose layer in the microfluidic chip. (**a**) Bright-field (*top*) and fluorescence images (*bottom*) of the source and sink channels (*top*) at different time. (**b**) Fluorescein diffusion profiles during the period of 1 h. (**c**) Image processing. (*1*) Original image, (*2*) image after enhancing contrast, (*3*) image after subtracting background, and (*4*) binary image

- Identify regions of interest, for instance, a monolayer of bacterial cells within an area of 1.5 × 1.5 mm across the two channels.
- Use both "MosaiX Acquisition" and "time-lapse" functions in the AxioVision software to set up acquisition parameters. This allows acquiring a tile of images over the area at each time point (e.g., every 10 min).

3.6 Image Analysis for Determination of Bacterial Growth Rates

The time-lapse images are processed to obtain the area of bacterial colonies that are originated from individual cells (Fig. 6c). From this the growth rates can be calculated by analyzing the area at various times (*see* **Note 18**).

- Enhance the contrast of the original images by using the "auto brightness and contrast" function in Image J.
- Subtract a level of background intensity from the images. This should preserve both the individual and colony features (*see* **Note 19**).
- Convert the image into black-and-white "binary" formatted images.
- Calculate the areas of each colony using the "analyzing particles" function in Image J. An example setting is the circularity

parameter of 0–1 and particle size from 0 to infinity. The value of the area will be given in terms of the number of pixels.

- Randomly select a number of colonies starting from single cells. The growth rates of the single bacterium within the colony can be calculated using the following equation:

$$\ln R = \ln(S/S_0) = \mu(t - t_m), \quad (1)$$

where R is the increment ratio, S_0 and S are the bacterial colony areas at the initial ($t = 0$) time and at time t, μ is the specific growth rate (h^{-1}), and t_m is the lag period of bacterial growth.

4 Notes

1. Needles can be used directly for connecting tubing with syringes. The ends of needles are cut off when they are used for connecting tubing into the chip. Alternatively, precut tubes can be obtained from the New England Small Tube Corporation.
2. Development time provided is an approximation since actual resist dissolution rates vary greatly under different agitations. White residues on the wafer surface after isopropyl alcohol rinsing indicate photoresist underdevelopment. Simply immerse the wafer in SU-8 developer for additional time and repeat the rinse step.
3. **Step 24** to **28** can be used for fabrication of silicon mold for the assembly chip.
4. Intimate contact should spread spontaneously without pressing of the PDMS chip. Several minutes cure in an oven promotes bonding between the two surfaces. If it is not possible to lift up the sides of the PDMS from the coverslip, then the bonding is sufficient. Do not apply excess pressure or curing time in oven during this step as this may result in irreversible channel collapse.
5. Both devices allow manipulation of the composition and spatial distribution of bacteria. The assembly device is an open platform. Technologies such as microarray and spotting that provide great flexibility in multiplexing and high spatial resolution can be employed. In the case of the enclosed devices, dynamic modulation of cellular composition can be realized during the process of on-chip assay which is not possible with the assembly device.
6. It is recommended to apply silicone adhesives around each needle and wait for 2 h to allow it to dry. This seals the inlet/outlet ports and prevents leakage in the case of high pressure inside the channels.

7. It is critical to avoid bubbles in the whole system. Bubbles are often trapped between the syringe and needle. It is essential to fill the end of the needle with liquid before bringing the two together. In the case that a bubble has been found in the channel, first check whether there are bubbles in any other part of the system (e.g., tubing, the connection between syringe and needle). If not, a higher flow rate can be used to remove bubbles.
8. The cell-loading process lasts for about 20 min. With more cells being loaded in the trapping chambers, the resistance of chambers increases gradually and can reduce cell-loading rates. (In this case, increase the flow rate slightly, but not too high in case it causes leakage). Cell composition and seeding density can be adjusted in situ. For example, several types of cells can be sequentially delivered into the trapping chambers to form a structured community.
9. Low flow rate is preferred since it uses fewer reagents and minimizes the convection flow inside the trapping chamber. However, the flow should be high enough to remove shed cells from the flow channel.
10. Electrically programmable valves (e.g., solenoids valves) can be also used.
11. It is difficult to eliminate convective flow across the trapping channels due to the instability of pumps and the formation of the bacteria layer.
12. In order to track single-cell growing, the cell density should be controlled between 0.05 and 0.08. The low density can avoid bacterial colonies merging together during long-period experiments.
13. If the plastic holder is tightened too much, it can break the agarose membrane and cause leakage.
14. It should be noted there is only molecular exchange between the solutions in the channels and the surrounding cells. No cells are removed from the chip. This is a limitation of the assembly devices. The flow rate of solutions in the channel doesn't have significantly effects on the exchange of the molecules since the process is driven by slow diffusion.
15. Fluorescein is used for characterization of the concentration gradient formation of small molecules, such as amoxicillin. This is because they have similar molecular weights and consequently similar diffusion characteristics. In the range of 0 to 30 mM, the fluorescence intensity of fluorescein has an excellent linear relationship to its concentration.
16. Stable flows in the two channels were found to be important to maintain the steady profile for the long period of experiments.

17. Because of manual assembly, slight differences may exist from time to time. Therefore, it is good practice to calibrate the concentration gradient using fluorescein solutions each time after the experiment finishes. Since stable gradients form after 20 min, the calibration time can be shortened to 1 h.
18. Since single bacteria grow as a non-confluent monolayer, cell mass in a colony can be considered to be directly proportional to the colony's area. At least 20 single-cell colonies should be randomly chosen for a particular concentration.
19. The level of background intensity subtracted depends on the images.

In summary, the microfluidic platforms described enable long-term, continuous culture and single-cell tracking under well-defined environments. The enclosed terrace device provides great flexibility and speed for modulating the surroundings of individual cells, including both bacterial species and soluble stimuli. The manually assembled microfluidic device offers a unique combination of easy implementation in the majority of microbiology labs, generation of stable concentration gradients, and thus fast quantification of a factor (e.g., an inhibitor) on cell growth. Importantly, the approaches described above to studying bacteria do not require labeling of cells and can create a biomimicking environment, thereby offering unique advantages in the investigation of naturally occurring microbes.

Acknowledgment

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Cultivating Fastidious Microbes

J. Cameron Thrash, Jessica Lee Weckhorst, and David M. Pitre

Abstract

Cultivation of microorganisms is necessary for testing hypotheses generated from cultivation-independent microbial community analysis and modern "omics" techniques, yet many of the organisms identified using these methods resist cultivation with enrichment-, selection-, or solid medium-based approaches. Success in isolating some of the "most wanted" microorganisms has come from dilution-to-extinction methodology in a high-throughput format which effectively isolates cells upon inoculation and allows for competition-free growth in sterilized natural milieu or defined media. This methodology has been revolutionary to studies of marine microorganisms, where many of the most abundant taxa are also very small, slowly growing cells that are adapted to low nutrient concentrations and frequently have complicated nutrient requirements that are not easily predicted from geochemical data. Coupling dilution-to-extinction inoculation with large numbers of wells and monitoring with highly sensitive flow cytometry provides a means to bring many important and elusive microbial taxa into the laboratory and thus support experimental investigation of their physiological capabilities.

Keywords: Artificial medium, Cultivation, Culturing, Flow cytometry, High-throughput culturing, Marine medium, Microbiology

1 Introduction

Cultivation of microorganisms has proved successful for a variety of important strains, particularly pathogens; however, it has also been apparent for over 100 years that only a fraction of the total number of microorganisms observed via direct microscopic counts were capable of being grown in the lab, a phenomenon described as "The Great Plate Count Anomaly" [1]. The advent of rRNA gene sequence taxonomy using environmental DNA was revolutionary in that it exposed the diversity of microorganisms present and helped establish the evolutionary relationships between them [2]. It also dramatically compounded the problem of The Great Plate Count Anomaly, because it revealed that many of the most abundant, and therefore potentially the most important, microorganisms on Earth were as yet uncultivated. One of the most famous

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examples of this was the discovery of the ubiquity and dominance of the marine bacterioplankton of the SAR11 clade [3, 4].

The application of next-generation shotgun sequencing to whole community DNA has dramatically expanded our understanding of the overall diversity of microorganisms and the diversity of genes within these organisms. Although reconstruction of microbial genomes directly from the environment was originally limited to low-diversity communities [5], the continuing decline of sequencing costs and significant improvements in short-read metagenomic assembly tools [6] have made it feasible to reconstruct whole metagenomes from individual taxa within highly complex microbial communities, thereby providing an almost complete picture of the genomic content of uncultivated organisms [7].

However, in spite of the observational successes brought about by meta-omics approaches (also including metatranscriptomics, metaproteomics, and single cell genomics), they remain measurements of microbial potential and are thus more hypothesis-generating, rather than hypothesis-testing, techniques. For example, although proteorhodopsin was discovered in metagenomic sequences and expressed in *E. coli* to confirm its activity, the function of this gene in native marine microorganisms remained elusive until a series of cultivation-based studies finally showed its role in alleviating energy starvation in some otherwise heterotrophic microorganisms, including SAR11 [8]. Elsewhere, researchers demonstrated an abundance of SAR11 genes for the metabolism of one-carbon and methylated compounds in metagenomic data, but only cultivation-based studies could reveal that these pathways were utilized for energy production but not for growth [9]. Given the significance of this organism in marine systems, such a finding has dramatic consequences for models of carbon turnover. Thus, microbiology still depends on systematic efforts to isolate important microorganisms for experimentation in the laboratory, many of which have resisted cultivation with common methods that have remained essentially unchanged since the nineteenth century.

As a result of the value of pure cultures, cultivation methodology and theory has continued to develop, and many highly abundant marine microorganisms, previously only known through cultivation-independent techniques, have been isolated in the last decade. Key advances came through the oligotrophy research and theories of Don Button and colleagues. Marine systems, particularly in open water, are frequently in a state of nutrient limitation, whether it be from iron, phosphorous, nitrogen, carbon, or other key compounds, such as vitamins. Oligotrophic microorganisms, broadly defined as those that grow in nutrient-poor environments (although sometimes defined as being unable to grow in nutrient-rich environments [10]), are specially adapted to extremes in nutrient limitation, a feature that has confounded cultivation efforts because most standard media have high nutrient concentrations and oligotrophs typically

have slow growth rates. Button and colleagues demonstrated success in cultivating oligotrophs through a combination of strategies: natural seawater media and dilution-to-extinction culturing [11].

The original ZoBell's Marine Medium 2216 contained nitrogen, phosphorous, iron, and carbon concentrations orders of magnitude above those measured in seawater [12]. Button's hypotheses about oligotrophy extended to transporter kinetics, surface-to-volume ratios, and biomass and growth-rate considerations [11, 13], wherein oligotrophs were modeled to have small cell size, smaller genomes, to be fastidious- dependent on uptake of particular combinations of exogenous compounds, rather than subsisting through *de novo* production. As such, sterilized natural seawater was used as a medium for isolation of some of the first oligotrophs, including *Cycloclasticus oligotrophus* RB1 [14].

Oligotrophs in general were also predicted to grow slowly, even in "optimal" conditions, because they are by definition adapted to utilizing low levels of nutrients and not capable of rapid boom and bust growth patterns. Observations of natural seawater with cultivation-independent methods have supported the conclusion that many of the most commonly cultivated marine microorganisms, such as *Alteromonas* strains, are in fact "weed" organisms, present naturally at low abundance, but capable of rapid growth blooms [15]. As a consequence, to avoid growth competition by such organisms, cultivation of oligotrophs has also utilized versions of dilution-to-extinction inoculation to isolate individual cells away from others [11, 16–19]. The current technique measures the number of cells in seawater through direct counts with microscopy or flow cytometry and then uses that value to guide the volume of seawater inoculum that should be added. Typical goals are to inoculate media with a volume that should yield 1–10 cells per well [17, 18, 20].

Giovannoni and colleagues developed the so-called high-throughput culturing laboratory (HTCL), involving microtiter plates and molecular screening for particular taxa, and extended the successes of Button and previous researchers to the isolation of dozens of new marine strains [16]. Over fifty of these have now been genome-sequenced [21], including diverse members of the *Bacteroidetes*, *Actinobacteria*, a novel phylum *Lentisphaerae*, and the *Gamma-*, *Beta-*, and *Alphaproteobacteria*, the most famous of which was *Candidatus* Pelagibacter ubique HTCC1062 (P. ubique), the first cultured representative of the globally dominant SAR11 clade [4, 22]. P. ubique was widely observed as the most abundant marine microorganism by cultivation-independent techniques for over a decade prior to its isolation and thus represents one of the principal successes of the HTCL approach. Cultivation of P. ubique has also revealed insights into the fastidious nature of this organism, information that was only obtained upon development of a defined medium [23]. Subsequent

continued efforts by Giovannoni, Rappé, and Cho, with improved automation via flow cytometry and variations in inoculum source, have provided additional SAR11 strains [17, 20, 21]. The HTCL approach also recently yielded the first successful isolation of a member of the SUP05/Arctic96BD-19 clade [18], organisms that have been identified as dominant members of oxygen minimum zones [24].

This protocol is based on the procedures developed in [16–18, 25] with modifications. It should be noted that due to the use of dilution-to-extinction methodology, more abundant organisms will be isolated with higher probability than those of lower abundance. However, depending on environmental conditions, these may be transiently abundant members of the normally rare biosphere. The overall method can be visualized in the steps summarized in Fig. 1. Sterilized natural milieu-based medium is highly effective for cultivating fastidious microorganisms, and we also supply a recipe for a complex yet defined marine medium, informed by detailed chemical data, to account for implementing the protocol far from marine sources and to eliminate the batch-to-batch variation that can

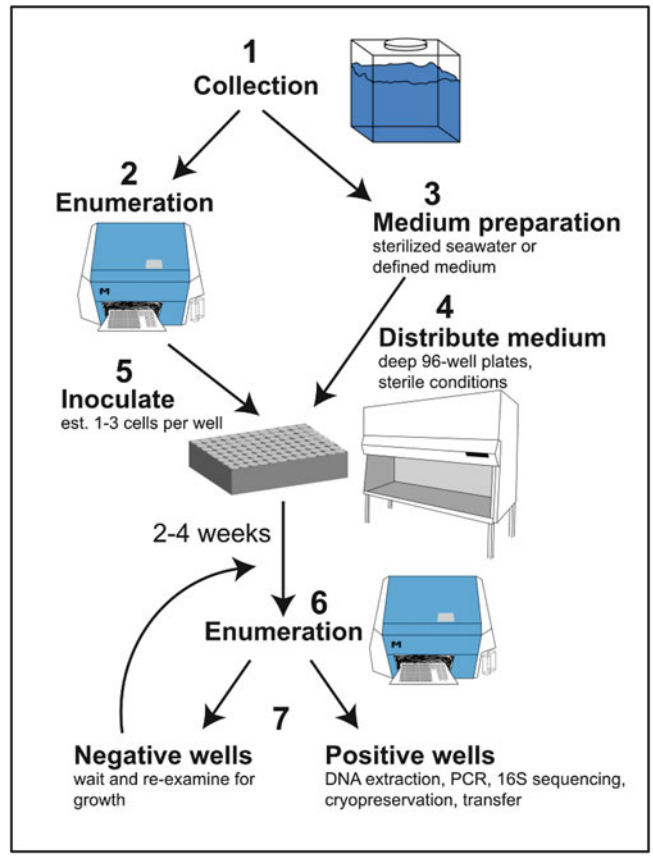

Fig. 1 High-throughput culturing flowchart

accompany natural media. Our modular recipe allows for customization of the different components, making it, and the complete protocol, easily adaptable to a variety of environments. For example, cultivation from freshwater or other fluid systems, including pore water, aquifer, or reservoir environments, should be possible as long as good data exists on the chemical makeup of the liquid such that defined media can be developed to mimic it or suitable quantities can be collected for sterilization and the fluid itself doesn't interfere with flow cytometry or other enumeration methods. Suggestions are included for modification of this method to accommodate such systems and others, including anaerobic environments and soil, and we include several other ideas to expand the usefulness of the protocol in Sect. 3.6. Additionally, we provide our protocols for enumeration and analysis of cultures using the Millipore Guava benchtop flow cytometer. These assume a basic understanding of flow cytometry, including the signal from the fluorescence and scatter parameters available on the Guava, and that users have already been properly trained on the equipment. For ease of interpretation, the Materials and Methods are organized with major subheadings coordinated according to Fig. 1.

2 Materials

2.1 Collection and Filtration

1. Carboys: 1 L polycarbonate carboys (Nalgene) for collection of natural inoculum, 20 L if seawater will be used as medium.
2. 10 % HCl in a large volume (~25 L) for acid washing of collection carboys, medium preparation bottles, and incubation flasks.
3. Whatman GF/D glass fiber filters (25 mm) and housings (*see* **Note 1**).

2.2 Enumeration

1. Guava 5HT HPL benchtop flow cytometer and associated computer/reagents (Millipore) (*see* **Note 2**).
2. SYBR Green (Life Technologies) diluted to 100× (from 10,000× stock) in TE for staining DNA of cells for enumeration (*see* **Note 3**).
3. Round-bottom 96-well plates (see Millipore Guava user manual for list of appropriate plates).
4. Live culture of an appropriate microorganism to serve as a positive control, e.g., *Escherichia coli*.

2.3 Medium Preparation

2.3.1 Sterilized Natural Medium

1. 20 L polycarbonate carboys (Nalgene).
2. 142 mm 0.2 μm Supor filters (Pall Life Sciences).
3. 142 mm filter housing (Geotech).
4. Peristaltic pump (Cole-Parmer).

5. Sterilization pouches (Fisher).
6. Compressed CO_2 and air, with associated regulators and storage space.
7. 0.2 μm in-line AcroPak 20 filters (Pall Life Sciences).
8. 0.1 μm in-line AcroPak 20 filters (Pall Life Sciences).
9. Masterflex tubing, 1/4" ID for sparging, 3/8" ID for filtering (Cole-Parmer).
10. Sparging apparatus. Construct double-vented carboy lids by drilling two holes in each lid, inserting plastic two-sided 1/4" barbed male hose fittings, and sealing the top side of the inserted fitting with super glue. Attach Masterflex tubing long enough to reach into the liquid volume to the bottom side of one of these fittings. Organize tubing with one in-line 0.2 μm AcroPak filter and one 0.1 μm AcroPak filter to the top side of the same lid fitting, 0.1 μm filter closest to the lid. Attach tubing with one in-line 0.2 μm air filter to the top side of the other fitting for venting. Place in a sterilization pouch.
11. Insert two-sided male barbed fitting in the tubing from the CO_2/air cylinders to connect to the sparging apparatus

2.3.2 Defined Medium

1. Complete lists of the defined medium components are in Tables 1, 2, 3, 4, 5, 6, and 7.
2. 0.2 μm sterile filters (Fisher) for filter-sterilization of trace metals and vitamin mix.
3. Pyrex screw-top bottles (Corning), 200 mL, 2 L
4. 50 mL conical tubes

2.3.3 Solid Medium

1. Agar (Fisher)
2. Plastic Petri dishes (Fisher)
3. Disposable inoculation loops (Fisher)

2.4 Medium Distribution

1. PTFE 2.1 mL × 96-well plates (Radleys, Essex) for incubation of the cultivation experiment (*see* **Note 4**).
2. PTFE-coated silicon 96-well plate mats (Thermo Scientific) for sealing the cultivation plates to prevent contamination and evaporation.
3. Laminar flow hood or biosafety cabinet for inoculation/transfer with reduced contamination risk (*see* **Note 5**). The hood is wiped down with 70% EtOH before and after use to improve sterility. UV light sterilization in between uses, if available, can be added according to the manufacturer's instructions.

2.5 Inoculation and Incubation

1. Sterile cabinet as in Sect 2.4(3).
2. Incubator capable of maintaining in situ temperatures.

Table 1
Basic marine medium components

	FW	g/L	Final M
Basic salts			
NaCl	58.44	23.84	408 mM
KCl	74.55	0.746	10 mM
$NaHCO_3$	84.01	0.840	10 mM
Na_2SO_4	142.04	4.27	30 mM
NaBr	102.89	0.0823	800 μM
H_3BO_3	61.83	0.026	420 μM
$SrCl_2$	158.53	0.0142	90 μM
NaF	41.99	0.0023	55 μM
KH_2PO_4	136.09	0.007	51 μM
Mg/Ca stock (20×)		g/100 mL	
$MgCl_2 \times 6\ H_2O$	203.30	21.2	52 mM
$CaCl_2 \times 2\ H_2O$	147.01	3.04	10 mM
Iron stock (1,000×)			
$FeSO_4 \times 7\ H_2O$	278.01	0.0028	101 nM
NTA Na_2 salt	235.10	0.0081	345 nM

Table 2
Trace metals (100,000 × stock)

Compound	FW	g/100 mL	Final M (nM)
$MnCl_2 \times 4\ H_2O$	197.91	0.018	9
$ZnSO_4 \times H_2O$	179.47	0.002	1
$CoCl_2$	129.84	0.001	0.5
Na_2MoO_4	205.92	0.001	0.3
Na_2SeO_3	172.94	0.002	1
$NiCl_2$	129.60	0.001	1

2.6 Large Format Enumeration

1. Multichannel pipette (Gilson) for efficient transfer of 200 μL volumes from 96-well incubation plates to 96-well counting plates.
2. Repeater pipette (Gilson) with 2 μL capability for rapid dispensation of SYBR Green into 96-well plates.

2.7 Positive Wells

1. DNA extraction (*see* **Note 6**).
2. 16S rRNA gene PCR (*see* **Note 7**).
3. 125 mL polycarbonate flasks (Fisher).
4. −80°C freezer or liquid N_2 dewar.

Table 3
Vitamin mix (100,000× stock)

Compound	FW	g/100 mL	Final M (nM)
B1/thiamine	337.27	1.69	500
B2/riboflavin	376.36	0.0026	0.7
B3/niacin	123.12	0.985	800
B5/pantothenate	238.27	1.013	425
B6/pyridoxine	205.64	1.028	500
B7/biotin	244.31	0.0098	4
B9/folic acid	441.40	0.0177	4
B12	1355.4	0.0095	0.7
Myoinositol	180.16	0.901	500
4-Aminobenzoic acid	137.14	0.0823	60

Table 4
Inorganic nitrogen mix (2,000× stock)

Compound	FW	g/100 mL	M in stock
$NaNO_3$	84.99	0.646	0.076
$NaNO_2$	69.00	0.028	0.004
NH_4Cl	53.49	0.053	0.01

Table 5
Misc C & N mix (2,000× stock)

Compound	FW	g/100 mL	M in stock
L-Glutamine	146.14	0.015	0.001
Dextrose	180.16	0.018	0.001
D-Ribose	150.13	0.015	0.001
Sodium pyruvate	110.04	0.011	0.001
Sodium citrate	294.10	0.029	0.001
Oxaloacetic acid	132.07	0.013	0.001
Sodium acetate	82.03	0.008	0.001
Sodium succinate	162.05	0.016	0.001
α-Ketoglutaric acid	168.08	0.017	0.001
Urea	60.60	0.061	0.01

Table 6
Fatty acid mix ($2\times10^6\times$)

Compound	FW	Density	g/100 mL	mL/100 mL	M in stock
Octanoic acid	144.21	0.91		15.85	1
Decanoic acid	172.26		17.23		1
Isobutyric acid	88.11	0.95		9.27	1
Butyric acid	88.11	0.964		9.14	1
Valeric acid	102.13	0.939		10.88	1
Ethanol	46.07	0.789		54.86	9.4

Table 7
Amino acid mix (50,000×)[a]

Compound	FW	g/L	M in stock
L-Arginine × HCl	210.70	6.32	0.030
L-Cysteine × 2HCl	194.16	1.56	0.008
L-Histidine × HCl × H_2O	209.65	2.10	0.010
L-Isoleucine	131.17	2.63	0.020
L-Leucine	131.17	2.62	0.020
L-Lysine × HCl	182.69	3.63	0.020
L-Methionine	149.21	0.76	0.005
L-Phenylalanine	165.19	1.65	0.010
L-Threonine	119.12	2.38	0.020
L-Tryptophan	204.23	0.51	0.002
L-Tyrosine	181.19	1.80	0.010
L-Valine	117.15	2.34	0.020

[a]Purchased from Sigma, cat# M5550, as 50× but added 20 μL/L

5. Cryovials and storage boxes (Thermo Scientific), glycerol (Sigma).
6. Glycerol stocks (20%–final concentration 10%) are prepared in a biosafety cabinet or laminar flow hood by combining 47.5 mL growth medium (3.3) and 2.5 mL glycerol in a bottle while stirring, filter-sterilizing into a conical vial, and distributing 200 μL to cryovials. These can be stored at 4°C until use (*see* **Note 8**).

3 Methods

3.1 Water Collection and Filtration

1. All culturing hardware (carboys, flasks, glassware, PTFE plates, sealing mats, stir bars) are washed first with lab-grade detergent; rinsed with hot tap water, cold tap water, and then DI water; and let dry. They are then soaked in a 10% HCl acid bath overnight, rinsed six times with MilliQ water, lids applied or wrapped in foil, and autoclaved prior to storage or use.
2. Collect samples at any source of interest with sterile, acid-washed carboys appropriate to the required volumes, taking as many precautions as practical to avoid contamination. Collectors should wear gloves. Samples should be transported back to the laboratory with the shortest delay possible to reduce potential bottle effects that can alter the community prior to inoculation [26].
3. Filter sample(s) prior to enumeration using a Whatman GF/D glass fiber filter to exclude large aggregates/particles and increase the proportion of planktonic cells in the seawater (*see* **Note 1**).

3.2 Enumeration of Samples Prior to Dilution and Inoculation

3.2.1 Preparation of Samples

1. Transfer 200 μL of each sample, in triplicate, into a round-bottom 96-well plate.
2. Transfer 400 μL of each of the desired controls into a 0.5 mL microcentrifuge tube (Sect. 3.2(2)). Typically, controls consist of stained and unstained medium and stained and unstained *E. coli* (*see* **Note 9**).
3. Stain samples and controls with 1× final concentration SYBR Green (2 μL 100× SYBR Green added to 200 μL sample). Incubate samples for 30 min in the dark.

3.2.2 Adjust Settings

1. The following is used with Incyte Software (version 2.7) for the Guava easyCyte HT systems from Millipore (Darmstadt, Germany). It also assumes that the Guava has been properly cleaned and that the EasyCheck protocol has been completed as recommended by the manufacturer.
2. Plots. Forward scatter (log scale) vs. green fluorescence (log scale), side scatter (log scale) vs. green fluorescence (log scale), and a five-decade histogram of green fluorescence are used to evaluate the counts.
3. Instrument settings need to be tailored by application and stain used, but we currently utilize the following gain parameters: FSC 1, SSC 2.83, GRN 4.56, YEL 8, RED 8.
4. Controls. Unstained controls (*E. coli* and artificial medium) are used to set a green fluorescence threshold for exclusion of autofluorescence and system noise, respectively. Stained

controls (*E. coli* and artificial medium) are used for gating results around standard noise of the artificial media and identify the potential area for positive cell growth (Sect. 3.2.4, below).

5. Adjusting settings. While running the control samples, the green fluorescence threshold is set to exclude noise based on the unstained controls (Fig. 2). These settings are saved and used for counting the samples (*see* **Note 10**).

3.2.3 Counting

1. A worklist for counting is set up according to the manufacturer's instructions.
2. Counting is set to acquire 3,000 events or last for 90 s, whichever comes first.
3. Control samples are counted along with the experimental samples.
4. Total cells μL^{-1} needs to be < 500 for a given sample to stay within the accurate counting range of the Guava. If samples are higher, make appropriate dilutions in sterile medium.

3.2.4 Analysis, Gating

1. Cell concentration is calculated using a gated region, which is selected based on the side scatter vs. green fluorescence plot. Forward scatter vs. green fluorescence can also be used but may reduce resolution depending on the sample (*see* Figs. 3 and 4, ungated examples).
2. Initially, a global gate is applied to plots using the stained artificial medium and stained *E. coli* samples as a reference such that the gate excludes the stained medium signal and includes the positive growth of *E. coli* (Fig. 5).

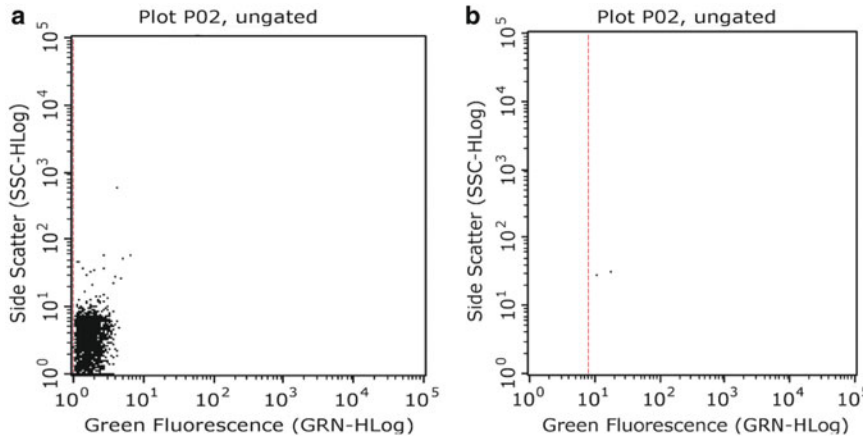

Fig. 2 A green fluorescence threshold is used to exclude system noise with unstained medium. (**a**) No threshold; (**b**) threshold applied (*red line*)

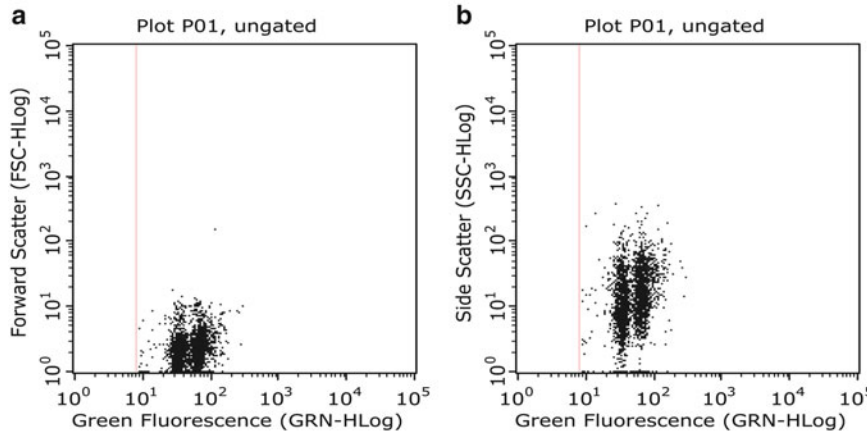

Fig. 3 Plots showing (**a**) forward scatter vs. green fluorescence and (**b**) side scatter vs. green fluorescence for a growing marine isolate

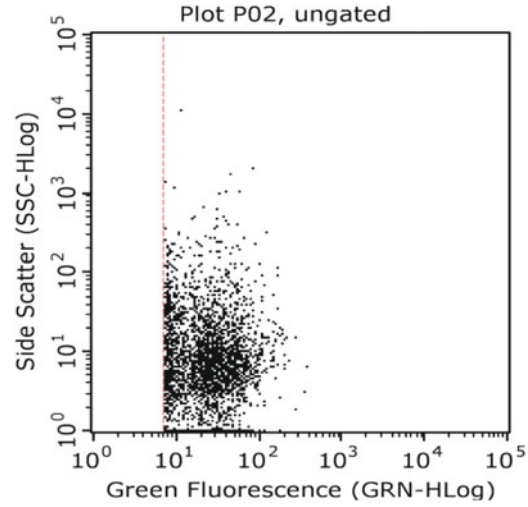

Fig. 4 Ungated plot of GF/D filtered seawater, 1:100 dilution

3. However, especially in the case of isolates (Sect. 3.3(6), below), but sometimes with whole seawater, individual samples must have the gate customized to account for variations in signal compared to *E. coli* or another positive control (Fig. 6).
4. In practice, this will be empirically defined, as different media will give different amounts of background noise.

3.3 Medium Preparation

3.3.1 Sterilized Natural Medium (Seawater)

1. This can be prepared according to the protocols in [16, 17] upon return to the laboratory after collection of large (20+ L) Volumes.
2. Filter the water sample using 142 mm 0.2 μm Supor filters and a peristaltic pump into another acid-washed clean carboy.

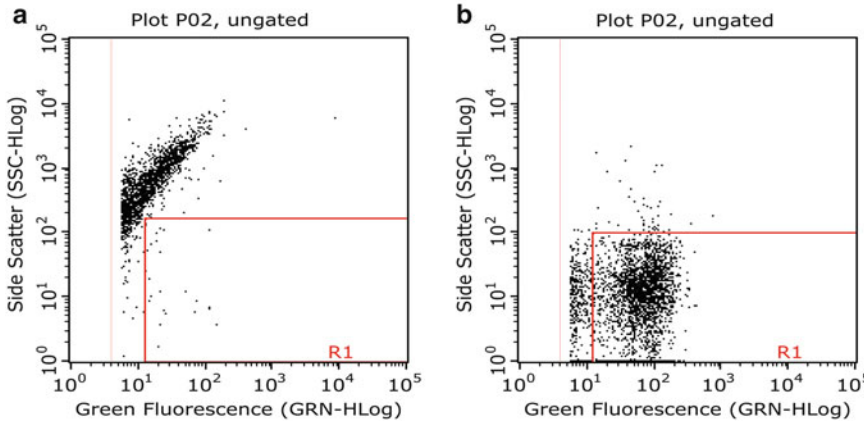

Fig. 5 Plots show gating around SYBR Green stained (**a**) artificial medium and (**b**) *Escherichia coli*

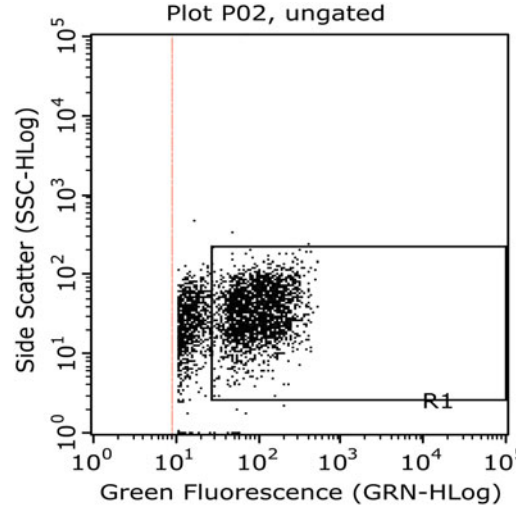

Fig. 6 A local gate is applied to a plot with positive growth of an isolate

3. Autoclave the filtered water with carboy lid loose and let cool. For 20 L, autoclave for 5 h and for 4 L, 2 h. To ensure sterility, cover the lid with a sterilization pouch and do not allow any water to splash to the level of the lid during transport post autoclaving.
4. Separately autoclave the sparging apparatus for 35 min in a sterilization pouch.
5. Place entire cool carboy and sparging apparatus in the biosafety cabinet or laminar flow hood, being careful not to splash the water up to the lid.
6. Attach autoclaved air sparging apparatus without touching internal tubing to anything during transfer into the carboy. Connect to cylinder tubing and sparge with CO_2 for 6 h and

then with air for 12 h. Adjust flow rate until bubbling in the medium is at a moderate pace without causing splashing.

7. Place carboy back in biosafety cabinet or laminar flow hood and exchange sparging apparatus for a newly autoclaved lid.

3.3.2 Defined Medium (Marine)

1. Can be prepared in advance according to Tables 1–5.
2. Dissolve reagents for the Mg/Ca stock and iron stock separately in 100 mL MilliQ-filtered water and autoclave.
3. Separately dissolve the trace metals and vitamin mix in MilliQ-filtered water as two separate solutions, 0.2 μm filter-sterilize, and aliquot into smaller volumes. Store at 4°C with vitamin mix wrapped in foil.
4. Dissolve reagents for the Misc C and N mix and inorganic nitrogen mix in appropriate amounts of MilliQ-filtered water as two separate solutions. Dissolve fatty acid mix components in ethanol using a fume hood. Filter-sterilize and aliquot into smaller volumes; store at 4°C.
5. Using a 2 L screw-top Pyrex bottle, combine basic salts together in 950 mL MilliQ-filtered water.
6. Add 10 μL trace metals to the Basic Salts.
7. Add carbon and nitrogen as desired to the basic salts (Tables 4, 5, 6, and 7). Our laboratory typically adds the inorganic nitrogen mix (Table 4) to a final N concentration of ~50 μM (consistent with our own Northern Gulf of Mexico/Mississippi River plume measurements and [27, 28]) and combines carbon mixes (e.g., Tables 5, 6, and 7) to result in a total final C concentration of ~50–100 μM (consistent with values such as those in [27]) (*see* **Note 11**).
8. Autoclave basic salts mix with cap tight to avoid loss of the bicarbonate buffer; cool to room temperature (RT) after autoclaving.
9. In the biosafety cabinet or laminar flow hood, while stirring, add 10 μL vitamin mix, 50 mL Mg/Ca stock, and 1 mL iron stock to the RT basic salts.
10. pH and record. pH should be ~8.2–8.3.

3.3.3 Solid Medium

1. The above media (3.3.1 and 3.3.2) can be used to make 1.5–2% agar plates.
2. In the case of the defined medium, 15–20 g L^{-1} agar is added to the basic salts and other components using 940 mL MilliQ-filtered water (instead of 950 mL) prior to autoclaving, omitting $NaHCO_3$, (*see* **Notes 12–14**).
3. Separately, make a 1 M solution of $NaHCO_3$ in MilliQ-filtered water, and 0.2 μm filter-sterilize.

4. After autoclaving, but above the gel point, add the Mg/Ca stock, vitamin mix, iron stock, and 10 mL of 1 M $NaHCO_3$ solution on a warm hot plate while stirring to prevent premature solidification.
5. Pour medium into Petri dishes roughly halfway full in a biosafety cabinet or laminar flow hood. Cover with lids and allow to solidify.
6. For natural medium, add agar prior to autoclaving, with addition of $NaHCO_3$ and plate pouring done in the same manner.

3.4 Liquid Medium Distribution

1. Medium is distributed into sterile, acid-washed deep 96-well plates (2.1 mL PTFE) at 1.7 mL per well in a biosafety cabinet using a sterile transfer pipette.
2. Wells are only filled with 1.7 mL to allow for a small headspace and the depressions of the sealing mats.

3.5 Inoculation and Incubation

1. All steps except incubation performed inside a biosafety cabinet or laminar flow hood.
2. Based on enumeration (Sect. 3.2, above), cells are diluted in medium such that they can be inoculated into deep 96-well plates using minimal volume. The average number of estimated cells per well should be 1–3 after inoculation. A typical inoculum has cells diluted to 0.5–1 $\times$ 10^3 cells mL^{-1}, and 2–3 μL is added per well.
3. Control wells at the four corners of each plate are left uninoculated to account for possible contamination during all procedures.
4. PFTE plates are sealed with sterile silicon sealing mats and placed in incubators at in situ temperatures in the dark (*see* **Note 15**).
5. Incubation is carried out for 2–4 weeks before the first enumeration (*see* **Note 16**).

3.6 Large Format Enumeration

1. Carried out in the same manner as Sect. 3.2 with the following adaptations for high-throughput counting with 96-well plates.
2. In a biosafety cabinet or laminar flow hood, using a multichannel pipette, transfer 200 μL from the deep 96-well incubation plates to 96-well round-bottom counting plates.
3. Using a repeater pipette, eject 2 μL 100× SYBR Green into each well of the counting plates without touching the liquid in any well with the pipette tip.
4. Since all 96 wells of the counting plate will be utilized, add stained/unstained medium and *E. coli* or other controls to 500 μL microcentrifuge tubes in the large volume section of

the Guava counting tray. Include these in your counting worklist.

5. For higher throughput, counting can be set to 3,000 events or 60 s (Sect. 3.2.3). This will facilitate counting multiple 96-well plates in a day with a large number of negative wells. Since the goal is to obtain simply a growth/no-growth result, rather than a highly accurate count of a low-density culture, longer count times are unnecessary to determine positive wells.

3.7 Evaluation of Results

1. Positive wells. Wells showing growth are split with portions transferred to large-volume incubations and cryopreservation:
   (a) The threshold for "positive" is 10^4 cells mL^{-1} (*see* **Note 17**).
   (b) When wells are deemed positive, 200 μL is transferred to duplicate glycerol stocks, allowed to mix, and cryopreserved by transfer to −80°C or liquid N_2 (*see* **Note 8**).
   (c) Medium is prepared according to Sect. 3.3 and 50 mL distributed in a biosafety cabinet or laminar flow hood to 125 mL polycarbonate flasks. Transfer 200 μL of culture to the flasks using a biosafety cabinet or laminar flow hood (*see* **Note 18**).
   (d) Upon growth to ~10^5 cells mL^{-1}, DNA extraction is performed on filtered cells or pelleted cells using any method desired (*see* **Note 6**). Additional samples ($n = 4$–10) are cryopreserved to increase culture security.
   (e) To identify isolates and provide a first assessment of culture purity, 16S rRNA gene PCR is completed on genomic DNA, and amplicons are sequenced (*see* **Note 7**).
2. Negative wells. Wells that show no growth may be reevaluated at later time points (*see* **Note 19**).

3.8 Downstream Steps to Consider

1. *Additional isolation measures.* Although dilution-to-extinction cultivation frequently results in axenic cultures, there is always the chance that two or more cells were added to any given well during inoculation. Therefore, we recommend that additional measures be taken to improve the chances of culture purity, such as repeated dilution-to-extinction assays and plating:
   (a) Dilution-to-extinction assays are completed in 96 × 2.1 mL plates, whereby a 1:100 inoculum of a growth culture is added to well 1 and mixed, and 10% of this well is added as inoculum (with a new pipette tip) to the next adjacent well, and so on for 10–12 wells. The most dilute well that shows growth is used as the inoculum for larger growth cultures. This process can be repeated serially to improve rigor.

(b) Plating. The solid medium recipe in Sect. 3.3(3) allows for plating of putatively pure cultures. Cultures are streaked on plates inside a laminar flow hood or biosafety cabinet using disposable loops and incubated under the same conditions as in the original isolation culture. Although growth on plates may not occur, if it does, not only is the isolation procedure made more secure and repeatable, additional studies requiring solid medium (e.g., motility studies) will now be possible.

2. *Additional glycerol stocks.* It is prudent to continue sequestering glycerol stocks for a culture upon successful large-volume propagation. This should also be done each time a frozen stock is revived to avoid as much genetic change in the culture as possible.

3.9 Suggestions for Modification to Incorporate Alternative Environments/Taxa

1. *Alternative natural liquid media.* The procedures for natural seawater medium above can easily be transferred to natural estuarine or freshwater medium, including lakes, streams, aquifer pore water, etc. Smaller volumes could also be utilized from such biological sources as tissue, liquid excrement, etc. if enough volume can be collected to inoculate a growth plate and some additional post-isolation growth flasks. In the case of small collection volumes, smaller volume (1 mL) deep well plates could substitute for the 2 mL plates. For liquids that are potentially not autoclavable, these could be 0.2 μm filter-sterilized prior to distribution. Sparging of such liquids as in Sect. 3.3(1) is unnecessary.
2. *Defined freshwater/estuarine medium.* The main constituents of the medium in Sect. 3.3(2) (Na, Cl, B, SO_4, Br, Mg, Ca, PO_4, and Fe) can be altered to mimic a variety of freshwater or estuarine environments, using the same basic strategy of matching concentrations with measured values. For example, the basic salts and Mg/Ca mix can be diluted with varying amounts of water to create different estuarine media. Chemical data such as in [29] can be used for altering the media to mimic particular estuarine environments.
3. *Substrate manipulation.* Because of the modular nature of the medium recipe, considerable customization can be done to select for organisms with particular capabilities. For example, selection for single carbon substrate utilizing organisms can be done by omitting the carbon mixes and substituting a single compound (such as acetate or a hydrocarbon like hexane) into the basic salts mix prior to autoclaving. Similarly, specific combinations of compounds can also be tested in this manner, similar to [23].
4. *Growth on solid medium.* Since many organisms require solid surfaces on which to grow, solid medium (Sect. 3.3(3)) may also be used to supplement the liquid cultivation process.

In this case utilization of sterilized natural milieu combined with a solidifying agent would still provide the benefits of medium mimicking the natural environment. To improve throughput to the level of the liquid method, and the odds of successful cultivation, it is recommended to prepare 100+ plates. GF/D filtered inoculum can be streaked on plates inside a laminar flow hood or biosafety cabinet. A filtered inoculum will help avoid plating particles and consortia with multiple organisms.

5. *Anaerobic growth.* This method can accommodate anaerobic cultivation with the use of an anaerobic growth chamber (Coy Laboratory Products, Inc.) and appropriate equipment for making anaerobic medium (e.g., see such studies as [30]). Once a sterile anaerobic medium is created, **steps 3.4** and **3.5** can be completed in the anaerobic chamber with clean 96-well plates that have been allowed to equilibrate overnight in the chamber to remove any remaining oxygen. For incubations in the dark, sealed plates can be wrapped in foil. Large format counting can be completed in the same manner as in 3.6, with the modification that the initial transfer to counting plates be completed in the anaerobic chamber. Fixation with formalin prior to staining with SYBR Green and after removal from the chamber would avert changes from exposure to oxygen during the counting procedure.
6. *Soil and other nonaquatic systems.* For nonliquid systems, the protocol can potentially still be utilized after mixing of a solid mass into a slurry with liquid medium. This slurry could then be filtered, counted, and diluted, with additional downstream steps remaining the same. For examples of slurries prepared from soil, plant leaves, and coral, see [31–33], respectively.
7. *Immiscible/volatile substrates.* Immiscible carbon sources or other growth substrates could still be utilized with deep 96-well plates; however, organisms growing on these will be found near or on the substances, thus necessitating mixing for resuspension prior to cell counting. In general, enumeration of particle-associated organisms with flow cytometry is challenging, and thus alternative methods, such as direct fluorescence-based microscopy counts, might be preferred. An example utilizing this enumeration technique in the same general workflow as above is detailed in [16]. The protocol may be adaptable to even the use of volatile substrates if the plates can be maintained in a chamber where the volatile compound can equilibrate between the headspace and the liquid culture. For example, the use of hydrogen as an electron donor can be accomplished in a typical anaerobic chamber.

4 Notes

1. GF/D filters or an equivalent. The exact pore size is up to the user and may be increased or decreased depending on the size fraction from which desired isolates are expected.
2. Flow cytometric methods are useful for very large format counting and high sensitivity, especially for small cells. However, versions of this protocol use direct enumeration with epifluorescence microscopy, which may be useful for those on a budget but with access to an appropriate microscope. This will substantially affect throughput.
3. SYBR Green has been used at higher concentrations in other protocols (e.g., [18]).
4. PTFE plates are optimal, due to their low binding capacity and reusability, but are also expensive and opaque. Other commercially available options may be more attractive for cost purposes and/or for translucence if cultivation of phototrophs is desired.
5. A biosafety cabinet is not necessary – a laminar flow hood can also be used.
6. Extractions can be completed using a variety of protocols. This step is at the user's discretion, but it is important to have a method which can successfully isolate DNA from cell densities at ~10^5 cells mL^{-1} or lower to avoid potentially lengthy growth optimization efforts for higher cell densities. Our laboratory uses the MoBio Power Water kit (MoBio).
7. Primers for 16S rRNA gene amplification are at the user's discretion as certain groups may be desirable for specific targeting. See [34] for an excellent assessment of optimal primers for this gene. We frequently use 27F/1492R (S-D-Bact-0008-d-S-20/S-*-Univ-1492-a-A-21 in [34]), but if amplification fails, additional primer sets should be deployed, particularly to account for Archaea.
8. Users should experiment with different concentrations of glycerol for a given culture. If storage capacity allows, initial freezing should be done with a range of final glycerol concentrations to ensure best recovery results.
9. Alternative positive controls that are more similar to the desired cells may be used, including other known isolates and fluorescent beads with similar sizes.
10. The threshold may change position based on media, as can be seen in the various figures, operationally ranging in our lab from 30 to 90.
11. Total N calculations must take amino acid and urea additions in the other mixes into account.

12. $NaHCO_3$ is left out at this stage because some will be volatilized during autoclaving, and the medium is not being allowed to cool in a closed container as in Sect. 3.3(2). Therefore, $NaHCO_3$ must be added after autoclaving to maintain a buffer.
13. For solid medium with agar, users may consider adding a separate solution of phosphate after autoclaving, rather than combining them prior. This has been shown to improve cultivation from natural communities [35].
14. Agar alternatives, such as gelatin, guar gum, etc., should be explored as possible substitutes as some organisms either are capable of utilizing the agar as a carbon source or cannot grow on this substrate.
15. Dark incubations are used to exclude the growth of phototrophs or mimic natural conditions where needed. Incubations that want to include phototrophs can be completed in the light and will require translucent plates (*see* **Note 4**, above).
16. This allows for slow-growing organisms to become numerous enough to be counted but can be tailored to in situ temperature. For example, typical Gulf of Mexico surface water temperatures in the summer are ~30°C, and thus incubations are expected to require less time than for those with temperatures 10 or 20°C cooler.
17. In practice, positive growth of pure cultures can usually be observed at 10^3 cells mL^{-1} with the Guava, but using 10^4 cells mL^{-1} provides a more conservative cutoff and is consistent with [17], which used the Guava technology as well.
18. Propagation of cultures to higher cell densities can frequently be accomplished by increasing the concentration of the carbon and/or nitrogen mixes, e.g., 2×, 5×, etc., over successive transfers.
19. Some organisms require longer adaptation times and/or have extremely low division times, and there is evidence that dormancy can play a role in cultivation success [36]. Thus, wells may also become positive after longer incubation periods regardless of division time.

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Cultivation and Preservation of Hydrocarbonoclastic Microorganisms, Particularly *Cycloclasticus* Species

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Abstract

Microbes capable of using hydrocarbons as carbon and energy sources are widely distributed in marine environment and play a significant role in the mitigation of petroleum pollution. For the majority of marine microbes, their isolation and long-term maintenance in the laboratory is difficult. Among the hydrocarbon-degrading bacteria, there is a specific group of organisms, mainly within the class of Gammaproteobacteria, whose metabolism is restricted to the aliphatic and/or (poly)aromatic hydrocarbons and their derivatives. This group of marine obligately hydrocarbonoclastic bacteria consists of the several genera, namely, *Alcanivorax*, *Cycloclasticus*, *Oleiphilus*, *Oleispira*, *Thalassolituus*, and *Oleibacter*. This chapter discusses the methods for isolation, cultivation, and preservation of these hydrocarbonoclastic bacteria. Many different types of hydrocarbon-degrading bacteria have been isolated from a range of marine environments using minimal media amended with nutrients and hydrocarbons under various incubation conditions.

Keywords: Cultivation, *Cycloclasticus*, Hydrocarbonoclastic bacteria, Marine oil pollution, Microbial degradation

1 Introduction

Even with a boom in metagenomic approaches applied to environmental samples, the methods of isolating, growing, and maintaining bacteria and/or consortia in the laboratory still have much to offer. Cultivation of microorganisms is essential in order to study their physiology, including establishing their organic substrate preferences and identifying metabolites. It also provides the principal means to discover novel enzymatic activities and new transformations [1]. Hydrocarbonoclastic bacteria (HCB) [2, 3] are abundant

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members of microbial communities associated to hydrocarbon-polluted marine areas [4]. The distribution and role of HCB during bioremediation have been extensively reported [5–12], and they also have a number of applications in blue biotechnology (e.g., novel enzymes, biosurfactants, bioplastics) and in natural attenuation of oil-polluted seawater.

Such applications generally require the cultivation of HCB strains for a number of specific reasons:

- To verify the degradation performance of cultures under both optimal cultivation conditions and in situ, which improves the ability to predict when and where HCB will bloom in the natural environment.
- To identify the environmental factors limiting the efficacy of HCBs, which would improve intervention strategies and emergency plans.
- To identify the HCB's key enzymes and secondary metabolites (e.g., biosurfactants), which have a key role during biodegradation. This allows exploitation of these products as an alternative approach, to the use of whole-cell biocatalysts, and provides another way to monitor biodegradation processes in real time.
- To select microbial consortia or pure strains suitable for a wide variety of applications. The high specialization of HCB let to hypothesize not only the implementation of bioremediation but also peculiar and unique molecular adaptation, which cannot be found in other microorganisms (e.g., synthesis of biosurfactants, bioemulsifiers, and amphiphilic siderophores).

Of the five typical HCB genera (*Alcanivorax* [13], *Thalassolituus* [14], *Oleiphilus* [15], *Oleispira* [16], and *Cycloclasticus* [17]), *Cycloclasticus* and *Oleiphilus* [18] species are the most difficult to cultivate. *Cycloclasticus* is the only PAH degrader within the marine HCB. Although the medium ONR7a was optimized originally to isolate *Cycloclasticus pugetii* [17], there are still challenges to maintain the strain in a viable and cultivable state and to grow it in liquid culture. We are still lacking complete understanding of the chemical, biological, and physical prerequisites needed for the trouble-free growth of *Cycloclasticus* sp. [19]. Active metabolism and multiplication can be inhibited by the presence of toxic products, especially for bacteria thriving in highly polluted environment [10, 20]. Regarding the habitats from where the strain has been isolated or detected, it is notable that members of the genus *Cycloclasticus* have been frequently found in oily sediments and petroleum-contaminated shallow and deep seawaters [21–23]. Moreover, there is evidence of their existence as the endosymbionts of mussels and sponges, as well as on the surface of the seep asphalts and in hydrocarbon seep [24, 25].

Liquid enrichments of *Cycloclasticus* spp., as many other marine bacteria, are frequently found in coculture with other bacteria

belonging to the genera *Rhodococcus*, *Thalassospira*, and *Marinobacter* ([23], Denaro et al. unpublished). Such co-occurrence is also documented for mixed enrichments where a functional synergy has been observed. In particular, Cui and coworkers [23] investigated the biodegradation of pyrene and fluoranthene by the defined coculture of *Cycloclasticus* sp. and *Marinobacter* sp. *Cycloclasticus* was not able to grow in axenic culture, whereas *Marinobacter* can grow separately only on several low molecular weight PAHs. In coculture, *Marinobacter* failed to grow on pyrene or fluoranthene, but may degrade the intermediates produced by strain *Cycloclasticus* sp. during high molecular weight PAH metabolism. This behavior can require close physical proximity of the two strains during isolation, as metabolites produced by one strain can help the other strain. In particular, *Cycloclasticus* sp. seems to lack the genes involved in iron uptake. This could lead to the hypothesis that it utilizes the iron-chelating siderophores produced by other neighboring bacteria, which promotes its growth in typically iron-impoverished marine habitats [26, 27].

2 Materials (*see* Note 4)

The information present here is intended to offer simplified guidance to materials needed to isolate, cultivate, and maintain marine obligately hydrocarbonoclastic bacteria in the laboratory.

2.1 Preparation of Culture Media

All products (salts, buffers, and solutions) necessary for the preparation of growth media may be supplied from any provider. Those frequently used in our laboratory are from Sigma-Aldrich and Difco.

1. ONR7a is the simplified artificial seawater medium [17].
   Composition per liter: Solution 1 (800 mL), Solution 2 (199 mL), and Solution 3 (1 mL)

   Solution 1: Dissolve 22.79 g NaCl, 3.98 g Na_2SO_4, 1.30 g TAPSO (3-[N-tris(hydroxymethyl) methylamino]-2-hydroxypropane-sulfonic acid) (*see* **Note 1**), 720 mg KCl, 270 mg NH_4Cl, 89 mg $Na_2HPO_4 \times 7\ H_2O$, 2.6 mg NaF, 31 mg $NaHCO_3$, 27 mg H_3BO_3, and 83 mg NaBr to 800 mL of distilled water; adjust pH to 7.6 + 0.2 at 25°C. Autoclave for 15 min at 15 psi and 121°C. Cool to 50°C before mixing with other solutions.

   Solution 2: Dissolve 11.18 g $MgCl_2 \times 6H_2O$, 1.46 g $CaCl_2 \times 2H_2O$, and 24 mg $SrCl_2 \times 6H_2O$ to 200 mL of distilled water. Mix thoroughly. Autoclave for 15 min at 15 psi and121°C. Cool to room temperature.

   Solution 3: Prepare 200 mg L^{-1} $FeCl_2 \times 4\ H_2O$ and sterilize by filtration through a Millipore membrane (0.22 μm). Store at room temperature in the dark.

For solid media, the following gelling agents can be used: either Bacto Agar (Difco) 15 g L^{-1} or Agarose (Sigma-Aldrich) 12 g L^{-1} or Gelrite™ (Adam Scientific) 8 g L^{-1}, by adding to Solution 1 before sterilization (*see* **Note 2**).

2. ONR8a medium: ONR7a supplemented with 10 mL L^{-1} of the vitamin solution (Table 1; [28]) and 10 mL L^{-1} of the modified ONR trace element solution (Table 2; [29]). Reduce the volumes of Solutions 1 and 2 to take account of these additions.
   2.a ONR8a + YE: ONR8a medium with addition of yeast extract (Difco) (50 mg L^{-1})
   2.b ONR8a + YEP: ONR8a medium additionally supplemented with yeast extract (50 mg L^{-1}) and peptone (Difco) 50 mg L^{-1} (*see* **Note 3**)
3. Aged and sterilized by filtration seawater supplemented with 1 mL of nitrogen/phosphorus (N/P) stock solution (*see* Sect. 2.1.1) [9]. Natural seawater is stored in the dark for at least 3 weeks to age (*see* **Note 4**).

   Storage of prepared media. The recommended shelf life of prepared culture media varies considerably. Screw-capped bottles of mineral broth can be stored for 6 months at low ambient temperatures (4–8°C). It is important to store all media in the dark. Agar plates should be stored at 2–8°C in sealed containers to avoid loss of moisture. Do not freeze.

2.1.1 Solutions for Media

1. Vitamin solution (Stock 100X).
   Prepare the vitamin solution (*see* Table 1).
   Sterilize by filtration with a Millipore membrane (0.22 μm) and add only when the medium is cooled to room temperature. Store in the dark in a refrigerator at 0–5°C. Precipitation in vitamin solutions may occur during storage. Do not use precipitated vitamin solutions (*see* **Note 5**).
2. Trace elements solution, modified for ONR medium (Stock 100X). In this case the composition of ONR trace element solution is similar to that used elsewhere [29] with some modifications: $FeCl_2$ is not used; the concentrations of both $Na_2MoO_4 \times 2H_20$ and $Na_2WoO_4 \times 2H_2O$ are correspondingly increased to 100 and 400 mg L^{-1} (Table 2).

   Sterilize by autoclaving for 15 min at 15 psi and 121°C. Store the solution at room temperature in the dark.
3. N/P Stock solution (100X; used for preparation of aged seawater medium): 10 g NH_4NO_3 and 2 g K_2HPO_4 dissolved in 100 mL distilled water, pH 7.6 and autoclaved for 30 min at 121°C.

2.2 Energy and Carbon Sources

1. Organic substrates: (*see* Sect. 3.2)
   Sodium acetate $Na(CH_3COO)$ (Sigma-Aldrich)

Table 1
The composition of Vitamine solution (100×)

Vitamin solution (100×), mg L^{-1}	
Biotin	2.0
Folic acid	2.0
Pyridoxine-HCl	10.0
Thiamine-HCl × 2 H2O	5.0
Riboflavin	5.0
Nicotinic acid	5.0
D-Ca-pantothenate	5.0
Vitamin B12	0.1
p-Aminobenzoic acid	5.0
Lipoic acid	5.0

Table 2
The composition of Trace element solution modified for ONR 8a (100×)

Trace element solution modified for ONR 8a (100×), g L^{-1}	
Nitrilotriacetic acid $N(CH_2CO_2H)_3$	1.50
$MgSO_4 \times 7\ H_2O$	3.00
$MnSO_4 \times H_2O$	0.50
NaCl	1.00
$FeSO_4 \times 7\ H_2O$	0.10
$CoSO_4 \times 7\ H_2O$	0.18
$CaCl_2 \times 2\ H_2O$	0.10
$ZnSO_4 \times 7\ H_2O$	0.18
$CuSO_4 \times 5\ H_2O$	0.01
$KAl(SO_4)_2 \times 12\ H_2O$	0.02
H_3BO_3	0.01
$Na_2MoO_4 \times 2\ H_2O$	0.10
$NiCl_2 \times 6\ H_2O$	0.03
$Na_2SeO_3 \times 5\ H_2O$	0.30
$Na_2WO_4 \times 2\ H_2O$	0.40

Sodium pyruvate $Na(C_3H_3O_3)$ (Sigma-Aldrich)
Sodium propionate $Na(C_2H_5COO)$ (Sigma-Aldrich)

2. Hydrocarbon substrates: (*see* **Note 6**)
Aliphatic hydrocarbons (e.g., hexane, heptane, octane, nonane, naphtha, petroleum naphtha, petroleum ether, tetradecane)
(Poly)aromatic hydrocarbons PAHs (purity of at least 99%) (e.g., naphthalene, phenanthrene, anthracene, pyrene, fluorene, fluoranthene, dibenzothiophene)
Volatile hydrocarbons (BTEX) (e.g., benzene, toluene, ethylbenzene, xylene)

2.3 Enrichments and Isolation of Hydrocarbon-Degrading Bacteria

1. For sample collection, containers made from high-density polyethylene (HDPE) or polytetrafluoroethylene (PTFE or Teflon®) are suitable for sample collection; brown borosilicate glass containers (or clear containers covered with aluminum foil) with PTFE-lined lids should be used for hydrocarbon analysis (*see* **Note 7**).
2. For enrichments, Erlenmeyer glass flasks (125- to 250-mL capacity) should be used.

2.4 Maintenance Procedures

Cryoprotective agents to store the hydrocarbon-degrading bacteria are typically used in concentrations ranging from 10 to 20% (v/v) (*see* Sect. 3.4):

1. Glycerol (Sigma-Aldrich ≥99%) has to be autoclaved (15 min, 121°C) and cooled before use. Store at room temperature in the dark.
2. Dimethyl sulfoxide $(CH_3)_2SO$ (DMSO) (Sigma-Aldrich ≥99%) has to be sterilized by filtration using a 0.22 μm nylon syringe filter or a Teflon® PTFE syringe filter, which has been prewashed with alcohol and rinsed with DMSO. Store at room temperature in the dark in glass or in the appropriate container provided by the manufacturer. DMSO is a cytotoxic agent, so handle with care (*see* **Note 8**).
3. Cryovials: Sterilize screw-capped 1.5-mL polypropylene vials.

3 Methods

This protocol describes detailed experimental procedure for isolation, culturing, and maintenance of HCBs. This method has been used successfully to isolate and maintain in the laboratory various HCBs including the problematic strains of the genus *Cycloclasticus*. In particular, we present here the protocol changes that enabled us to isolate and maintain axenically numerous strains of *Cycloclasticus*.

The isolation and growth of hydrocarbon-degrading bacteria typically follows enrichment in minimal liquid medium supplemented with an appropriate (mixture of) hydrocarbons as the carbon sources, due to the typically low abundance of HCB in non-contaminated seawater. Selection of hydrocarbon substrates and the method to increase their availability is critical to achieve new hydrocarbon marine degradators. Bacteria can be grown in both liquid or on agar-solidified media. Cultivation on agar plates (Petri dishes) is usually applied to facilitate the obtaining of single colonies.

To maintain an adequate supply of oxygen, cultures of aerobic microbes need to be shaken vigorously, leaving a large head space in the flasks.

3.1 Preparation of Culture Medium

1. ONR7a [7] is an artificial seawater used as the minimal mineral medium. This medium contains all major cations and anions that are present in concentrations greater that 1 mg/L in average seawater. Nitrogen is provided in the form of NH_4Cl and phosphorous is provided in the form of Na_2HPO_4. This medium was used for the first time for isolation of *Cycloclasticus pugetii*, a key obligate marine PAH-degrading bacterium [7].

   An important point, which should be taken into consideration during the preparation of ONR7a medium, is to avoid precipitation of salts. This is achieved by preparing and autoclaving separately Solutions 1 and 2 (Sect. 2.1). The solutions should be mixed only after they have been cooled to at least 50°C. Solution 3, containing oxidized Fe(II), has to be made as a 1,000 times concentrated stock and 1 mL added to one liter. For solid ONR7a medium, either 15 g L^{-1} of Bacto Agar or 12 g L^{-1} of Agarose (final concentration) has to be added to the Solution 1 prior to autoclaving (*see* **Note 9**).
2. ONR8a medium: In some cases modified ONR medium is used. In contrast to classical ONR7a medium, ONR8a is supplemented with 10 mL L^{-1} of the vitamin solution (Table 1) and 10 mL L^{-1} of ONR-modified trace elements solution (Table 2; *see* Sect. 2.1.1).
3. Aged and sterilized seawater supplemented with nutrients [9] is an another option to obtain the initial enrichment of hydrocarbon-degrading consortia. In this case, the trace elements are already present in aged seawater, but it is still necessary to provide the sources of nitrogen, phosphorous, and iron in amounts similar to ONR7a medium (Sect. 2.1.1).

3.2 Preparation Carbon Sources

1. When using organic acids: Pyruvate, propionate, and acetate (all in the form of sodium salts) dissolve 5 g in Solution 1 and autoclave (121°C, 15 min). Final concentrations of 5 g L^{-1} can be altered depending the oligotrophy of the HCB isolates, and

Table 3
Nonaromatic carbon sources utilized by selected *Cycloclasticus* strains

Carbon source	*C. pugetii*	*Cycloclasticus* N3	*Cycloclasticus* G
D-Glucose	–	–	Nd
D-Fructose	–	–	Nd
D-Mannitol	–	Nd	Nd
DL-Malate	–	Nd	Nd
α-Ketoglutarate	–	Nd	nd
Salicylate	+	–	–
Benzoate	+	nd	nd
Acetate	+	+[a]	+[b]
Propionate	+	+[b]	+[b]
Glutamate	+	+[a]	–
Succinate	Nd	+[a]	+[b]
Pyruvate	–	–	+

Cycloclasticus pugetii and C. N3 were isolated from Puget Sound sediments; strain G from Gulf of Mexico sediments
[a]Growth occurred when carbon source was provided at 0.1% but nota t 0.01% w/v
[b]Growth occurred when carbon source was provided at 0.01% but nota t 0.1% w/v

in case of cultivation of *Cycloclasticus* species, the concentrations of organic additives should be in a range of 1–0.1 g L^{-1} (Table 3).

2. Aliphatic hydrocarbons should be filter-sterilized with Millipore membrane 0.22 μm and afterward added to the combined medium (*see* **Note 9**). In solid medium, alkanes from *n*-decane (C_{10}) to *n*-hexadecane (C_{14}) can be added to a Whatman paper disk and placed on the lid of the Petri dishes. Alkanes with more carbon atoms are solids and then can be added as fine crystals (*see* **Note 10**).
3. For PAH substrates: Use a stock solution of PAH mix or the single substrate. For a PAH stock solution, phenanthrene, fluoranthene, pyrene, chrysene, or benzo[*a*]pyrene should be dissolved in an organic solvent (acetone, ethyl ether, or chloroform). The solutions can be sterilized by filtration through a 0.22 μm pore solvent-resistant membrane (e.g., polytetrafluorethylene (PTFE)). The solutions should be stored in a tight-capped glass bottles and kept away from the light and heat. PAH stock has to be added to empty flasks, the solvent must completely evaporate, and afterward the liquid medium is added, followed by an inoculum (*see* **Note 11**). In agar medium

PAHs can be supplied as fine crystals or dissolve each PAH independently into solvent (acetone) to 1–2% (w/v) final concentration and sprayed on the inverted upper lid.

4. Volatile hydrocarbons such as BTEX should be added to the medium separately after autoclaving and cooling down. They can be sterilized by filtration through a 0.22 μm membrane (e.g., PTFE) (*see* **Note 12**).

3.3 Enrichment and Isolation Hydrocarbon-Degrading Bacteria

In this section we describe a standard technique routinely used in our laboratory to select the most important marine obligate hydrocarbonoclastic bacteria (Sect. 3.3.1). Special emphasis is given to the enrichment and isolation of *Cycloclasticus* spp. (Sects. 3.3.2 and 3.3.3).

3.3.1 Conventional Methods for Seawater and Sediment

1. A small quantity of the sample (up to 5 g of marine sediments or 5 mL of seawater) is added to ONR7a artificial minimal medium (100 mL) in a sterile cotton-plugged 250-mL Erlenmeyer flask. Optionally prepare in duplicate.
2. Controls should be set up in parallel with added sediment or seawater but without additional carbon source.
3. The concentration of the relevant hydrocarbon for microbial growth should be in the range of 0.5–3.0 g L^{-1}, typically 1.0 g L^{-1} (*see* **Note 13**).
4. Enrichments can be continuously maintained in the laboratory by transfer of the late log-phase grown culture to fresh medium (1–10% [vol/vol]).
5. Enrichments should be incubated at temperatures corresponding to in situ conditions on an orbital shaker (100–200 rpm) in the dark (to avoid new primary production, but this may remove a favorable algal partner).
6. The growth times vary widely and may last from a few days up to several weeks depending on the type of microorganism, temperature, and the nature of the growth substrate.
7. Take a small quantity of enrichment (approx. 5 μL) (*see* **Note 14**), dilute in 50 μL of sterile minimal medium, and streak onto ONR7a agar plates amended with same substrate used in enrichment.
8. Incubate the plates at the selected temperature.
9. When colonies become visible, start to isolate pure cultures.
10. Identify the bacterial isolates.
11. Preserve purified strains (*see* Sect. 3.4).

3.3.2 Enrichment and Isolation of Cycloclasticus sp.

Enrichment from seawater:

1. Place 100 mL of oil-polluted seawater sample into a 500 mL baffled flask and directly add nutrients, namely, 100 mg

NH_4NO_3, 20 mg of K_2HPO_4, 2 mg of ferric citrate, and 100 mg of 2-methylnaphthalene or phenanthrene.

2. Incubate at temperature 20°C ±1 in a rotary shaker operating at 90 rpm for at least 4 weeks until the development of a yellow, orange, or a red-brown color (*see* **Note 15**).
3. Select positive enrichments and dilute serially with ONR7a medium (to 10^{-7} or 10^{-8}) and cultivate in medium supplemented with 1 g L^{-1} of phenanthrene.
4. Incubate until the development of a color, which typically would require about 2–3 weeks.
5. Spread the most diluted colored culture onto ONR7a plate overlaid with an opaque layer of 0.8% Agarose containing 1 g L^{-1} of phenanthrene (*see* **Note 16**).
6. Select the colonies around which is a zone of clearing on the opaque surface and transfer onto a new ONR7a plate supplemented with approximately 3 mg of phenanthrene placed on the lid of the plate.
7. Select colonies, purify by repeated plating, and proceed for further taxonomical analyses.

Enrichment from sediments:

1. Prepare a slurry with 10% (wt/vol) of oil-polluted sediments in ONR7a medium.
2. Place in a 1,000 mL-flask 200 mL of sediment slurry and add 10 ppm (2.0 mg) of phenanthrene for initial enrichment. Add the substrate periodically (approx. every 7 days).
3. Incubate for 4 weeks.
4. Prepare serially diluted cultures (up to 10^{-7} or 10^{-8}) in ONR7a medium and incubate as in the point 2.
5. Proceed as from the point 5 of the "Seawater" section.

Alternatively, enrichments can be prepared as follow:

1. Place 10 g of polluted sediment and 100 mL of seawater from the same site in a 1,000 mL flask.
2. Dilute 1:3 with ONR7a amended with 10 ppm of naphthalene and 10 ppm of phenanthrene
3. Incubate in a rotary shaker until the development of color.
4. Proceed as in the section "Sediments."

3.3.3 Alternative Approach to Enrichment and Isolation of Cycloclasticus sp.

1. Mix a portion of 10 g of hydrocarbon-polluted sediments in a Waring blender for 1 min at a power setting of 50%.
2. Dilute the supernatant 1:5 with ONR7a and mix well.

3. Spread 200 μL of the resulting preparation onto ONR8a + YE agar plate (*see* Sect. 2.1).
4. Place approximately 200 mg of biphenyl on the Petri dish lid.
5. Incubate the inverted plate for 3–4 weeks. Periodically look for the development of a diffusible yellow zone surrounding the colonies.
6. Transfer positive colonies on a new plate containing ONR7a medium using the same substrate (in this case biphenyl) as sole source of carbon.

3.4 Maintenance Procedures

For future studies and biotechnological applications, strains and/or consortia of marine obligate hydrocarbonoclastic bacteria need to be maintained and preserved over extended periods.

The majority of HCB has been successfully preserved at −80°C for several years. The operation includes several steps:

1. Preparation of culture: Grow a fresh culture in an appropriate liquid medium (*see* **Note 17**).
2. Preparation of cryoprotective agent: Glycerol and/or DMSO (*see* Sect. 2.4).
3. Add an appropriate volume of cryoprotective agent (final concentration 10–20% v/v) to the bacterial suspension placed in the sterile cryovials.
4. Invert the tube several times or vortex vigorously.
5. Store for 2–3 h at −20°C.
6. Store at −70 or −80°C for long-term preservation.
7. Prepare replicates (minimum duplicates, better triplicates, or more). Store in separate boxes in different freezers.
8. To revive a stored strain, streak onto appropriate medium (*see* **Note 18**).

4 Note

1. The use of certain PAH substrates could require an increase in the TAPSO concentration to 25 mM.
2. The solidifying agents used instead of the agar serve to reduce the carbon sources present in the medium. In particularly, agents such as Gelrite™ (gellan gum cab). This highly purified polysaccharide (formerly known as PS-60 and S-60) displays several interesting properties, including an extremely low concentration of organic contamination.
3. For the initial isolation of (poly)aromatic hydrocarbon-degrading bacteria, small amounts of yeast extract, peptone,

trace minerals, and vitamins should be added to ONR7a. Upon successive isolation, these supplements might be omitted if possible. However care must be taken to ensure that isolates degrade the PAH.

4. It is preferable to use the artificially prepared media rather than natural seawater to provide a reproducible solution of known composition and to avoid the negative effect of unknown compounds present in natural sample.
5. The precipitate can be dissolved by warming the solution for a short period of time in a water bath (35–37°C).
6. Take precautions when handling these chemicals, especially PAHs, which are highly toxic and often classified as carcinogens. Use with adequate ventilation and personal protective equipment.
7. During transportation to the laboratory, samples should, if possible, be kept cool and in the dark. Best practice is to process the sample within 48 h. Nevertheless, the samples could be also stored at 4°C for up to 1 month.
8. DMSO is highly effective in promoting "vitrification" (formation of a viscous glassy aggregate state of water without formation of damaging ice crystals) of the cells, but at the same time, this agent is cytotoxic. Therefore, it is better to use DMSO at concentrations not exceeding 15% (vol/vol).
9. Include a magnetic stirring bar in the medium when autoclaving so that it can be mixed on a stirrer prior to pouring into sterile plastic Petri dishes.
10. High molecular weight alkanes longer than hexadecane can be added to the medium directly and sterilized.
11. The choice of solvent used to dissolve PAHs is very important. All of these organic solvents are toxic and can destroy the cell membranes. They have to be removed by evaporation before addition of medium and inoculum.
12. When using the volatile organic compounds (benzene, toluene, and xylene), the application of plastic Petri dishes should be avoided, because these chemicals can deform the plastic. It is more suitable to use the glass Petri plates.
13. The concentration of the hydrocarbons used as sole carbon and energy source depends on their solubility, availability, and eventual toxicity.
14. Depending on the optical density reached in liquid cultures, plate 20–100 μL of appropriately diluted cell suspension onto agar plates.
15. The development of a yellow, orange, or red-brown color on PAHs indicates the ring cleavage and formation of intermediate

chromatic metabolites. 20°C is the temperature that has proven useful for most *Cycloclasticus* spp. isolated to date.

16. In this method we prepare a 0.8% Agarose containing crystals of phenanthrene (1 g L^{-1}); this is then poured onto the surface of an already solidified agar underlayer medium. The surface of the plates is opaque and it is visible that a clear zone forms around colonies of PAH-degrading bacteria.
17. For those strains that are not capable of growing in liquid medium, the colonies should be aseptically scraped off from the agar plate and resuspended in 0.7 mL of sterile medium and then follow the **steps 2–8** of Sect. 3.4.
18. In particular, while taking the inoculum from the glycerol stocks, do not thaw the whole cryotube. Keep the tube on ice and take a small sample with a sterile inoculation loop from the uppermost part of the frozen solution. After this, the tube can be frozen again in the −80°C freezer.

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Cultivating Aerobic Hydrocarbon-Degrading Bacteria from Micro-algae

Tony Gutierrez

Abstract

Over the past three decades, we have dramatically increased our knowledge on the types of bacteria playing key roles in the degradation of oil hydrocarbons in the ocean. However, there remains a lack of complete understanding regarding the full diversity of species contributing a role in hydrocarbon degradative processes. Recent evidence highlights the cell surface, or 'phycosphere', of micro-algae as a new and largely underexplored biotope in the ocean that harbours novel taxa of hydrocarbon-degrading bacteria, including obligate hydrocarbonoclastic bacteria (OHCB). OHCB are a 'specialised' group exhibiting an almost exclusive requirement for hydrocarbon substrates as a sole source of carbon and energy. Several novel species of OHCB, including one new family, were recently discovered from laboratory cultures of marine micro-algae and more likely await discovery. The protocol below provides a detailed description for isolating these types of organisms from marine micro-algae based on their ability to degrade volatile low-molecular-weight hydrocarbons, aliphatic hydrocarbons, and/or polycyclic aromatic hydrocarbons.

Keywords: Hydrocarbon-degrading bacteria, Marine environment, Micro-algae, Polycyclic aromatic hydrocarbons

1 Introduction

Bacteria play an important role in the degradation and ultimate removal of petrochemical pollutants from the marine environment. Over 20 genera distributed across the major bacterial classes (*Alpha-*, *Beta-*, and *Gammaproteobacteria*; *Actinomycetes*; *Flexibacter-Cytophaga-Bacteroides*) contain representatives of hydrocarbon-degrading bacterial species. Interestingly, a subset of these organisms exhibit the distinct capacity for utilising hydrocarbons almost exclusively as a sole source of carbon and energy. These hydrocarbon 'specialists', or obligate hydrocarbonoclastic bacteria (OHCB), appear to be solely confined to the marine environment – i.e. they are found nowhere else on earth. The importance of OHCB to help purging the oceans and seas is evidenced in the wealth of reports documenting their enrichment from near

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undetectable levels to constituting up to 70–90% of the total bacterial community at oil-impacted sites ([1, 2] and references therein). Of the currently established genera of OHCB, most comprise members that specialise in the degradation of linear or branched saturated hydrocarbons (*Alcanivorax*, *Oleiphilus*, *Oleispira*, *Thalassolitus,* and *Marinobacter*), whereas two genera, *Cycloclasticus* and *Neptunomonas*, comprise species that specialise in the degradation of polycyclic aromatic hydrocarbons (PAHs).

The ecology and life cycle of OHCB in the ocean, such as their possible interactions with and close dependence on other organisms, is an area that is in a nascent phase of understanding. It is in part this gap in our knowledge that is precluding our discovery of many more novel taxa of OHCB. Another reason is the recalcitrant nature of most microorganisms (>99%) to be amenable to cultivation in the laboratory. A common misconception when isolating bacteria from seawater is that they are thought to have been occurring in a free-living state, or merely no attention is given to the possibility that they may have been associated, or attached, to other planktonic surfaces. Aside from the many salts and dissolved inorganic and organic carbon (DIC, DOC) that constitute seawater, the ocean also contains enormous quantities of transparent exopolymer (TEP), particulate organic matter (POM), and an abundance of planktonic eukaryotic organisms (e.g. micro-algae), all of which can act as a surface from which bacteria can acquire nutrients as well as refuge. Improving our understanding on the ecology of OHCB, including niche sites where these organisms may be found to reside in the expanse of the ocean, could help in the design of targeted approaches for isolating these types of organisms, in turn uncovering more novel species and expanding our knowledge of their diversity and catabolic potential.

Recent evidence points to the cell surface layer, or phycosphere, of marine micro-algae (eukaryotic phytoplankton) as an underexplored biotope in the ocean for discovering novel OHCB [3–7], as well as other 'generalist' hydrocarbon degraders [8]. This algal–bacterial association may be attributed to the potential for some micro-algae to actively synthesise hydrocarbon molecules [9, 10] and translocate them to the algal cell wall [10–13] or to passively adsorb them from the surrounding seawater [14, 15]. Either through biogenic synthesis or adsorption of hydrocarbon molecules, the algal cell surface may be a 'hot spot' for attracting and the colonisation of hydrocarbon-degrading bacteria.

In this chapter, a method is described for isolating hydrocarbon-degrading bacteria (including OHCB) from marine micro-algae. The method targets bacteria that are capable of growing on and degrading saturated hydrocarbons or PAHs (e.g. *n*-hexadecane and phenanthrene, respectively, as model hydrocarbon substrates) as a sole source of carbon and energy.

2 Materials (*see* Note 4)

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 ONR7a Medium

1. Stock solution A: Dissolve 146 g NaCl, 39.8 g Na_2SO_4, 13.0 g TAPSO (*see* **Note 1**), 7.2 g KCl, 2.7 g NH_4Cl, 0.89 g $Na_2HPO_4.7H_2O$, 0.83 g NaBr, 0.31 g $NaHCO_3$, 0.27 g H_3BO_3, and 26 mg NaF in approx. 700 ml of water; adjust pH to 7.9; and then make up to a final volume of 1 l and autoclave (121°C, 15 min) (*see* **Note 2**).
2. Stock solution B: Dissolve 55.9 g $MgCl_2.6H_2O$, 7.3 g $CaCl_2.2H_2O$, and 0.12 g $SrCl_2.6H_2O$ in approx. 400 ml of water and then make to a final volume to 500 ml and autoclave (121°C, 15 min).
3. Stock solution C: Dissolve 11.8 mg $FeCl_3.6H_2O$ in 500 ml of water and autoclave (121°C, 15 min) (*see* **Note 3**).
4. 5 M NaCl stock solution: Dissolve 29 g NaCl in 100 ml water and autoclave (121°C, 15 min). Store at room temperature.
5. Trace element stock solution: Dissolve 4.36 g Na_2EDTA, 3.15 g $FeCl_3.6H_2O$, 0.022 g $ZnSO_4.7H_2O$, 0.01 g $CoCl_2.6H_2O$, 0.18 g $MnCl_2.4H_2O$, and 6.3 mg $Na_2MoO_4.2H_2O$ in 100 ml of water and filter sterilise (0.2 μm). Store at 4°C.
6. 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 water and filter sterilise. Store in the dark at 4°C.
7. Marine supplement stock solution: Combine 1 ml of the trace element stock solution, 5 ml of the vitamin stock solution, and 94 ml of water; autoclave (121°C, 15 min) or filter sterilise. Store at 4°C.
8. Bacteriological powdered agar: Purchased from most suppliers of microbiological media, such as Thermo Scientific Oxoid.

2.2 Carbon Substrates

1. Pyruvate, acetate, propionate (*see* Sect. 3.2).
2. Aliphatic hydrocarbons, such as *n*-hexadecane and *n*-tetradecane (*see* Sect. 3.2).
3. PAH substrates (>99% purity), such as naphthalene, phenanthrene, anthracene, pyrene, fluorene, fluoranthene, dibenzothiophene (*see* Sect. 3.2).
4. Benzene, toluene, xylene, phenol (*see* Sect. 3.2).

2.3 Enrichments for Isolating Hydrocarbon-Degrading Bacteria

1. Non-axenic algal strains can be obtained from most culture collections. My lab often purchases strains from the Culture Collection of Algae and Protozoa (http://www.ccap.ac.uk) and the Provasoli-Guillard National Centre for Marine Algae and Microbiota (http://ncma.bigelow.org) (*see* **Note 4**; *see* Sect. 3.3).
2. A vortex device: most common laboratory types can be used with a speed range of 200–3,200 rpm (*see* Sect. 3.3).
3. A temperature-controlled rotary shaker incubator: any conventional model can be used as long as it can accommodate glass Erlenmeyer flasks (*see* Sect. 3.3).
4. Erlenmeyer glass flasks (125- to 250-ml capacity): purchased from most suppliers of laboratory equipment (*see* Sect. 3.3).
5. Glass test tubes: purchased from most suppliers of laboratory equipment (*see* Sect. 3.3).

2.4 Isolation and Storage of Strains

1. For streaking: an inoculating loop and Bunsen burner (available from most suppliers of laboratory equipment).
2. For spraying PAH substrates onto the surface of agar plates: dissolve each PAH independently into acetone, ethyl ether or dichloromethane to 1–2% (w/v) final concentration. Store in the dark and ideally in solvent-tolerant spray bottles.
3. Autoclaved phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per 1 l, pH 7.5 (*see* Sect. 3.4).
4. Glycerol: >99% purity for long-term storage of isolated bacterial strains (*see* Sect. 3.4).
5. Cryovials: for long-term storage of isolated bacterial strains, we use screw-capped sterile 1.2-ml polypropylene vials (*see* Sect. 3.4).
6. A −80°C freezer: for long-term storage of isolated bacterial strains (*see* Sect. 3.4).

3 Methods

The following protocol describes a targeted approach to isolate hydrocarbon-degrading bacteria from micro-algae, although the method could be applied for isolating these types of bacteria from almost any environment. The method has been used successfully to isolate OHCB and generalist hydrocarbon degraders, including relatively fastidious strains that may not be easily amenable to cultivation in the laboratory that utilise other types of media or approaches. Although crude oil, or a consolidated hydrocarbon mixture, could be used to enrich for these types of bacteria, the

method described here uses a single hydrocarbon substrate per enrichment experiment. This single-hydrocarbon approach is preferred because hydrocarbon mixtures can otherwise cause competitive inhibition [16], which could potentially increase the likelihood of missing out on enriching, and subsequently isolating, some types of hydrocarbon degraders. Moreover, the use of a single hydrocarbon substrate per enrichment can enhance targeting for the isolation of certain hydrocarbon-degrading groups (respectively, aliphatic, monocyclic aromatic, and polycyclic aromatic degraders), which could be suppressed via competitive inhibition when using hydrocarbon mixtures.

3.1 Preparation of ONR7a Medium

ONR7a medium is described by Dyksterhouse et al. [17] (*see* **Note 5**). To prepare complete ONR7a broth, autoclave (121°C, 15 min) 670 ml of water first and then to this add 100 ml each of stock solution A, B, and C and 28 ml of the 5 M NaCl – ideally perform these steps in a laminar-flow cabinet to reduce the risk of contamination. Store at 4°C. When you are ready to use this medium, aseptically aliquot out the volumes that you require and add 5 ml of the marine supplements per litre of ONR7a – do this aseptically and store the remaining ONR7a at 4°C.

For ONR7a agar, add 15 g Bacto-agar to the 670 ml of water prior to autoclaving (*see* **Note 6**). Soon after taking the agar out of the autoclave, follow all the steps above for making complete ONR7a broth. You may need to reheat the medium at this stage if it has cooled too quickly. Use the stir bar to mix the agar prior to pouring into sterile plastic Petri plates. If making up ONR7a agar amended with either pyruvate, acetate, or propionate, add 10 ml of a 10% stock solution per litre of medium (equates to a 0.1%, final concentration) prior to pouring.

To make ONR7a agar plates amended with an aliphatic hydrocarbon substrate (e.g. *n*-hexadecane), vigorously vortex 10 ml of the selected hydrocarbon (presterilised by filtration; 0.2 μm) in a sterile 50-ml Falcon tube containing about 30 ml of acetone. Immediately after autoclaving the agar and mixing all the components for making complete ONR7a agar, vortex the hydrocarbon–acetone mixture and add it immediately to the ONR7a agar and mix well. Note that the role of the acetone is to act as a carrier for delivery of the hydrocarbon into the melted agar.

3.2 Preparation of Carbon Sources

1. Pyruvate, acetate, or propionate (sodium salt form) stock solution (10% w/v): Dissolve 10 g in 100 ml water and autoclave (121°C, 15 min). Store at room temperature (*see* Sect. 2.2).
2. Stock solution of aliphatic hydrocarbon substrates (e.g. *n*-hexadecane): Filter sterilise a neat aliquot (0.2 μm). Store at room temperature.

3. Stock solution of PAH substrates, each prepared individually (*see* **Note 7**): 3 g/l in 100% acetone; for naphthalene, however, 9 g/l in 100% acetone (*see* **Note 8**).
4. For enrichment experiments with volatile hydrocarbon substrates, *see* Sect. 2.3.

3.3 Enrichments for Isolating Hydrocarbon-Degrading Bacteria from Micro-algae

The methods described below for isolating hydrocarbon-degrading bacteria from micro-algae are presented using 250-ml glass Erlenmeyer flasks containing 50 ml of ONR7a and a select hydrocarbon substrate to commence the enrichments. However, most types of glass culture vessel that can be closed with autoclavable plastic or metal caps, or with cotton wool or foam plugs, will suffice. If using screw caps, be sure to keep the caps loosely fitted during incubation in order to allow for the diffusion of air and keep the culture well aerated.

3.3.1 Targeting Strains that Degrade Volatile Low-Molecular-Weight Hydrocarbons

1. To enrich for strains that utilise volatile hydrocarbons, insert a test tube inside each Erlenmeyer flask prior to autoclaving. After autoclaving, aseptically dispense 50 ml of ONR7a medium into each flask.
2. Inoculate the flask with a growing culture of the micro-algal strain to be used (ca. 10^6–10^7 cells per litre).
3. Aseptically dispense 1–3 ml of the hydrocarbon substrate into the test tube located inside the flask. The test tube acts as an isolated reservoir for the volatile substrate within the flask in order to deliver the substrate into the medium via the vapour phase, as previously described [18] (*see* **Note 9**; Fig. 1). The volatile hydrocarbon substrates my lab generally uses are benzene, toluene, xylene, pentane, hexane, phenol, biphenyl, and naphthalene (*see* **Note 10**), but others could be used depending on the types of hydrocarbon degraders being targeted.
4. The volatile hydrocarbon inside the test tube may need to be replenished periodically (approx. every 2–3 days), depending on the duration and temperature of the incubations, as its volume will be observed to diminish over time due to its volatilisation (*see* **Note 11**).
5. Incubate the enrichment cultures on a shaker (150–250 rpm), in the dark and at a selected temperature. Generally, an incubation of 1–2 weeks is sufficient, but longer times may be needed if low incubation temperatures (e.g. <10°C) are used which generally slow down microbial activity and require more time.
6. Take approx. 5 μl samples from each enrichment incubation and streak onto ONR7a agar glass plates (*see* **Note 12**).
7. Incubate the plates in a glass desiccator that accommodates a lid with an air vent – if the hole of the vent is >2 mm, plug it loosely with some glass fibre wool – to allow for the exchange

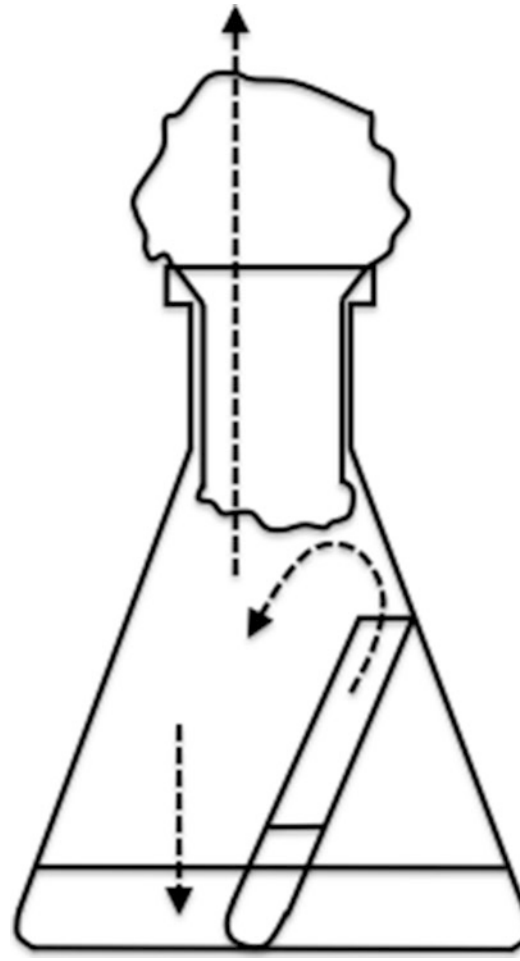

Fig. 1 Schematic representation of an Erlenmeyer flask setup used for enriching bacteria that degrade volatile hydrocarbons. *Arrows* indicate the movement of the volatile hydrocarbon from inside the test tube to the flask headspace and into the liquid medium and outside of the flask. A constant gas exchange is maintained between the outside air and headspace

of air to inside the desiccator, and the volatile hydrocarbon vapours to escape. This helps reduce exposure of the bacterial cells on the agar plates to very high and potentially toxic concentrations of the volatile hydrocarbon (*see* **Note 11**).

8. When colonies become visible, commence to isolate pure cultures of isolated colonies (*see* Sect. 3.4).

3.3.2 Targeting Strains that Degrade Aliphatic Hydrocarbons

1. Aseptically dispense 0.25 ml of filter-sterilised hydrocarbon substrate (e.g. *n*-hexadecane) to the bottom of an empty and autoclaved Erlenmeyer flask.
2. Aseptically dispense 50 ml of ONR7a medium.

3. Inoculate the flask with a growing culture of the micro-algal strain to be used (1–2 ml will suffice per 50 ml culture).
4. Incubate the enrichment as per **step 5** of Sect. 3.3.1.
5. Take approx. 5 μl samples from the enrichment incubation and streak onto ONR7a agar plates amended with *n*-hexadecane (*see* Sect. 3.1).
6. Incubate the plates at an appropriate temperature.
7. When colonies become visible, commence to isolate pure cultures of isolated colonies (*see* Sect. 3.4).

3.3.3 Targeting Strains that Degrade Higher-Molecular-Weight PAHs

1. Aseptically dispense a volume of the PAH (from a stock solution; *see* Sect. 3.2) to the bottom of an empty and autolclaved Erlenmeyer flask – my lab generally adds approx. 0.5 mg of the hydrocarbon.
2. Allow the acetone (or other solvent if used to prepare your PAH stocks) to volatilise off completely (*see* **Note 13**).
3. Aseptically dispense 50 ml of ONR7a medium.
4. Inoculate the flask with a growing culture (mid to late exponential phase) of the micro-algal strain to be used (1–2 ml will suffice per 50 ml culture).
5. Incubate the enrichment as per **step 5** of Sect. 3.3.1. Over the course of the incubation, periodically observe for the development of a change to the colour of the medium (*see* **Note 14**).
6. Take approx. 5 μl samples from the enrichment incubation and streak onto ONR7a agar plates.
7. Spray the surface of the agar plates with the same PAH substrate used in the original enrichment from step 1 (*see* Sect. 2.4) as per the method of Kiyohara et al. [19] (*see* **Note 15**).
8. The plates can be stored in closed plastic bags in the dark at room temperature.
9. When colonies become visible, select only those displaying clearing zones (Fig. 2) and proceed to isolating pure cultures (*see* **Note 16**; *see* Sect. 3.4).

3.4 Isolation and Storage of Strains

1. Transfer selected colonies to fresh ONR7a agar plates (amended or exposed to the same hydrocarbon substrate) and incubate under the same conditions as the original plate.
2. Once pure cultures are obtained, grow each strain in ONR7a broth amended with the hydrocarbon substrate used to isolate the respective strain (*see* **Note 17**).
3. Aliquot 0.3 ml of prewarmed autoclaved glycerol into a sterile Cryovial and allow to cool.

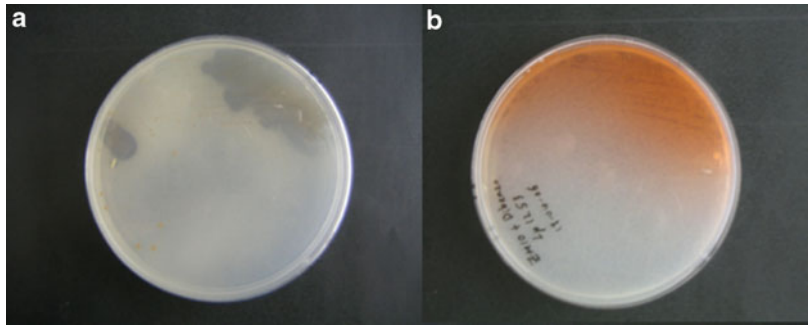

Fig. 2 Formation of clearing zones on ONR7a agar plates sprayed with phenanthrene (**a**) and dibenzothiophene (**b**). After streaking the plates, a thin film of the PAH substrate is sprayed onto the surface of the agar using an atomiser. Clearing zones are denoted by areas where the white crystalline PAH film has become dissolved on the agar surface, due to the activities of PAH-degrading bacteria localised within the cleared area. The formation of a diffusible pigment – often *yellow*, *orange*, or *pink* – is an indication of the formation of coloured intermediates from the degradation of the PAH substrate

4. Aliquot 0.7 ml of cell suspension above the glycerol and then vortex vigorously to mix (30% v/v/glycerol, final concentration).
5. Store the glycerol stocks at −80°C.

4 Notes

1. TAPSO and EDTA could potentially act as a source of carbon for some bacteria, so keep in mind that any isolated strains would need to be evaluated to sufficiently confirm they are capable of growing on and degrading any of the hydrocarbons tested. This could be done by, for, e.g. analysing for the disappearance of the hydrocarbon substrate using some analytical method, such as HPLC, GC, GC-MS or spectrophotometry.
2. pH is adjusted using a concentrated solution of NaOH. Do not store solution A in the fridge because some of the salts will precipitate out. Store at >10 °C, although room temperature should be fine.
3. If otherwise using the tetrahydrate form ($FeCl_2.4H_2O$), then adjust the amount to be dissolved in 500 ml accordingly – i.e. 10 mg.
4. Strains of non-axenic micro-algae can also be sourced from various other culture collections around the world. Upon receipt from the supplier, each strain can be maintained in algal medium that is specific to its respective growth requirements and grown in temperature-controlled illuminated incubators, per the supplier's guidelines. Instructions on how to isolate micro-algal species from environmental samples can be

found online or handbooks dealing with micro-algal culturing and maintenance (e.g. [20]).

5. The recipe for ONR7a is also described by the German Collection of Microorganisms and Cell Cultures (DSMZ) and is known as DSMZ Medium 950. However, Medium 950 does not appear to include the trace elements and vitamin mixture (i.e. for making the marine supplements), which is desirable for growing some fastidious strains.
6. Add a stir bar inside the bottle prior to autoclaving so that in the post-autoclaving stage, when all the components have been mixed for complete ONR7a agar, you can set the bottle on a magnetic stirrer to homogeneously mix the medium prior to pouring into plates.
7. Methanol or dichloromethane can also be used as alternative solvents, but not all PAH compounds, such as anthracene, readily dissolve in these solvents. It is important that the choice of solvent used is highly volatile because its purpose is to act as a carrier to deliver the PAH into the culture flask; the solute is allowed to volatilise completely before adding the medium and performing the inoculation.
8. To calculate the final concentration prepared for each PAH stock, make dilutions of each of your stocks using the same solvent (e.g. acetone) and measure these by UV spectrophotometry in quartz cuvettes; use the same solvent as the blank. Measure the absorbance using the maximum absorption wavelength (λ_{max}) for the respective PAH compound being measured. Then use the Beer–Lambert equation with the appropriate molar absorptivity coefficient (ε) specific to the PAH compound you are measuring [21] in order to calculate the concentration of the PAH in your stock; take into account the dilution you used; it is advisable to only use dilutions that give absorbance measurements between 0.1 and 0.8 in order to stay within the limits of reasonably accurate readings.
9. Take care not to accidentally dispense any of the volatile chemical into the liquid culture as these types of low-molecular-weight volatile hydrocarbons (e.g. benzene, toluene, xylene, phenol) are highly toxic at low concentrations.
10. Naphthalene is a solid PAH that is often supplied as granulated or fine powdered crystals. Use a small sterile spatula to transfer a few crystals to inside the test tube. The naphthalene vapours will diffuse into the culture medium. Same applies when using phenol.
11. Take precaution when using volatile chemicals as they are highly toxic and often classed carcinogenic. Locate your incubations either inside or near a fume hood or near an exhaust vent.

12. Do not use plastic Petri plates when exposing them to volatile hydrocarbons, such as the benzene, toluene, and xylene, as these chemicals can cause the deformation or melting of the plastic. Therefore, only use glass Petri dishes when making ONR7a agar that is to be used to enrich for volatile hydrocarbon-degrading strains.
13. Keep the cap on the flask slightly loose so that the solvent can escape whilst keeping the inside still sterile and not risk it becoming contaminated. It is important to make sure all of the solvent has volatilised (usually takes 12 h) as any residual solvent left inside the flask can cause some of the salts from the ONR7a medium, when the latter is subsequently added, to precipitate out of solution and result in a slightly cloudy-white medium.
14. The development of a yellow, orange, or pink colour in the culture medium is an indication for the formation of colorimetric dihydrodiol or quinone intermediates from the biodegradation of PAHs.
15. Working inside a fume hood, remove the lid of the streaked agar plate and position it upright so that the agar faces you. Then use an atomiser to spray the PAH on the surface of the agar. The solvent will be observed to volatilise off almost instantaneously, leaving behind a thin layer of the PAH chemical on the agar surface. Immediately place the lid back on the plate. The whole process should be performed expediently in order to minimise potential contamination in the fume hood.
16. The formation of clearing zones is an indication that they contain bacteria with PAH-degrading activities that have oxidised the PAH substrate and converted it from a crystalline (solid) state to a soluble form. One or more bacterial colonies may be found growing within the clearing zone and should be subcultured to isolate pure cultures (*see* Sect. 3.4). Sometimes a clearing zone may appear with no visible growth (or colony) associated within it, but it should nonetheless be treated as containing PAH-degrading bacteria and attempts progressed to isolate pure cultures from it. A colony with no apparent associated clearing zone likely represents a non-PAH-degrading strain; however, it may otherwise represent a strain that is a PAH-degrader that catabolises the respective PAH at a very slow rate.
17. If any particular strain is found unable to be grown in ONR7a amended with a hydrocarbon substrate, you could try to see if it grows on an intermediate of central metabolism, such as pyruvate, acetate, or propionate (*see* Sect. 3.2). For quite fastidious strains that are not amenable to growth in liquid medium, aseptically scrape off a loopfull of cells from the surface of the agar and suspend in 0.7 ml of sterile PBS and then follow **steps 3–5** of Sect. 3.4.

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Two-Phase Cultivation Techniques for Hydrocarbon-Degrading Microorganisms

Lukas Y. Wick, Sally Otto, and Christof Holliger

Abstract

In the following chapter, we discuss the role of two-phase substrate transfer and partitioning for substrate bioavailability. We describe four different two-phase cultivation techniques, which aim at controlling growth-limiting substrate mass transfer rates (Protocols 1 and 2) and/or at decreasing the toxicity of substrates (Protocols 3 and 4).

Keywords: Bioavailability, Chemostat, Cultivation, Mass transfer, Microorganisms, Partitioning, PDMS, Toxicity

1 Introduction

Actively growing microbes are typically surrounded by water. Their interaction with hydrocarbons and other hydrophobic compounds involves the water phase as they typically take up chemicals as water-dissolved molecules [1, 2]. The aqueous concentration of a chemical is generally taken as an indicator for its bioavailability for microorganisms, which is used to refer to *the degree of interaction of chemicals with living organisms*. Any crystalline, liquid, gaseous, nonaqueous phase liquid (NAPL)-dissolved or sorbent-bound hydrocarbons remain thus unavailable to catabolically active organisms unless they are transferred to the aqueous phase surrounding the microorganisms. Microbial growth on and transformation of hydrophobic compounds are thus often challenged by sufficient substrate delivery to the microorganisms, both during the biotechnological production of hydrocarbon-based intermediates or bioremediation approaches.

In this context, it is likewise important to distinguish between bioavailability for microbial degradation and bioavailability for microbial inhibition/bioaccumulation. In other words, whereas substrate addition at too low mass transfer rates will cause the cells to starve and result in suboptimal growth, the addition of

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the substrate at too high a concentration may inhibit or even kill the substrate-consuming organisms [3].

As microbial growth on hydrocarbons is a consumptive process, the bioavailable compound concentration is the result of a transient steady state that is governed by hydrocarbon uptake and hydrocarbon reprovision [4, 5]. At high mass transfer rates, microbial growth and hydrocarbon transformation are controlled by the metabolic activity and the population density of the bacteria. However, when transfer rates decrease or when the microbial population and concomitant substrate consumption grows, mass transfer becomes the growth-limiting factor, a fact often resulting in linear growth.

The situation is different for a microbe that is subject to the toxic effects of a bioaccumulating compound without having the possibility to degrade it. Here, an equilibrium situation will be approached. Unlike the steady state observed for biodegradation, the end point of bioaccumulation is characterized by a relatively high (freely dissolved) aqueous equilibrium concentration and zero mass transfer.

This situation gets even more complicated when cells are exposed to mixtures of substrates containing both beneficial and toxic compounds and when they require concentrations of a substrate at levels below its aqueous solubility for optimal growth [6]. In such cases, a precise and controlled delivery of substrates and/or nutrients is indispensable and has to be balanced (or adjusted) with the microbial growth rates.

Tailor-made control of the mass transfer fluxes of hydrocarbons can be effectively obtained by two-phase partitioning bioreactors [3]. These reactors are normally based on the use of a water-immiscible and biocompatible second (liquid, solid, sorbent, or gas) phase that is in contact with the cell-containing aqueous phase. The nonaqueous phases may either be the carbon source itself or it may dissolve/absorb large amounts of the substrate, which partitions into the aqueous phase at mass transfer fluxes determined by the experimental set-up. The advantage of such a system is that substrate supply – within the limits given by the interphase mass transfer constraints – is entirely driven by cellular processes. Additionally, the second phase at ideal situations may also function as a sink for toxic metabolites and foster optimal microbial growth.

Two-phase cultivation techniques are also applied if the solubility of the substrate is high enough to cause toxic effects but too low to allow exponential growth during longer periods in a batch culture. Conversely, a single bacterium can be encapsulated in a suitable second-phase matrix (e.g. alginate beads) to provide beneficial (non-toxic) nutrient and substrate conditions, e.g. for the cultivation of the uncultured [7].

1.1 General Approach

A useful way to conceptualize the transfer of a hydrocarbon from a nonaqueous to a (stirred) aqueous phase is by considering the interface between them as being composed of serially aligned mass transfer barriers [8] within which hydrocarbon transport

takes place by the relatively slow process of molecular diffusion. The overall transfer velocity (v_{tr}) of a chemical from a nonaqueous to the aqueous phase hence will be controlled by the diffusivity of the hydrocarbon in the interfacial boundary layers and their thickness. The mass (Q) of a hydrocarbon entering the cell-containing aqueous phase per unit of time (t) and a given interfacial area (A) can be described by

$$Q/t = A^{*}v_{tr}{}^{*}\text{Driving force} \quad (1)$$

Whereas the impact of the interface area A on Q is intuitively intelligible, the transfer velocity (v_{tr}) can be conceptualized as the ratio between the effective diffusivity of the hydrocarbon and the thickness of the diffusion-controlled interfacial boundary layers (i.e. an inter*ph*ase rather than an inter*f*ace as the latter would refer to an infinitely sharp plane between two phases) as explained above. Finally, the driving force is positively correlated to the difference in the chemical potential of the compound in the aqueous and nonaqueous phase [9]. Reference to Eq. (1) clarifies that any alteration in one of the above-described factors results in changed hydrocarbon mass fluxes into the aqueous phase and thus has a knock-on effect on microbial growth. As hydrocarbon transfer to the aqueous phase is proportional to the thickness of the diffusion-controlled inter*ph*ase layer, attachment of cells to second-phase hydrocarbons and subsequent biofilm formation is an efficient microbial adaptation to promote substrate bioavailability. Attachment increases the aqueous concentration-gradient and subsequently enhances microbial growth rates [10, 11] on liquid, solid, and NAPL-dissolved, absorbed, or surface-adsorbed substrates. Furthermore, experimental changes of the interfacial area, the hydrodynamic mixing, or the physico-chemical nature of the second phase and the concomitant effective diffusivity are useful parameters to specifically control two-phase cultivation [12, 13].

In the following, we describe exemplarily four two-phase cultivation protocols which aim at controlling growth-limiting substrate mass transfer rates (Protocols 1 and 2) and/or at decreasing their toxicity (Protocols 3 and 4). Protocol 3 describes microbial growth under anaerobic conditions and shows that two-phase cultivation is independent of the terminal electron acceptor.

2 Materials

Please note that the composition of the cultivation media depends on the organisms chosen and hence will not be specified further. Standard reagents and glassware specified below can be purchased, e.g. from Sigma-Aldrich (http://www.sigmaaldrich.com) and VWR International (http://vwr.com).

2.1 Protocol 1: Cultivation on Solid, Poorly Water-Soluble Substrates

Standard cultivation glassware and a gyratory shaker of known shaking velocity (VWR International (http://vwr.com)) are required.

2.2 Protocol 2: Cultivation Using a Polymer-Based Controlled Hydrocarbon Release System

Standard cultivation glassware and commercially available poly (dimethylsiloxane) (PDMS) (Altec Cornwall, UK; https://www.altecweb.com/) that can be sliced into pieces of an appropriate shape and surface area are required.

2.3 Protocol 3: Cultivation on Potentially Toxic Substrates

Bottles of known volumes tightly sealed with poorly absorbing Viton® stoppers in screw caps or crimp caps (e.g. from VWR International (http://vwr.com)) are recommended. Poorly biodegradable hydrocarbons are suitable for use as the organic second phase. Hexadecane can be used if microbial degradation is extremely slow or even not very probable (e.g. under anaerobic conditions). Otherwise poorly biodegradable heptamethylnonane is recommended.

2.4 Protocol 4: Cultivation on Substrates Provided via the Gas Phase

Petri dishes and desiccators are needed for cultivation on agar plates. If enrichment and cultivation in liquid medium is performed, small volume chemostats with pumps allowing operation at low dilution rates ($\leq 0.0075\ h^{-1}$) and mass flow controllers allowing controlled addition of the volatile substrates are required. For cultivation in liquid culture batch cultures, glass Erlenmeyer flasks with baffles, a sidearm, and with gas-tight septa and screw caps are suggested.

3 Methods

3.1 Protocol 1: Cultivation on Solid, Poorly Water-Soluble Substrates

This procedure aims at controlling the substrate fluxes to metabolically active cells of poorly water-soluble substrates such as the polycyclic aromatic hydrocarbon, anthracene ($c_{w,sat} \approx 40\ \mu g\ L^{-1}$). At sufficiently high aqueous concentrations, microbial growth is controlled by the metabolic activity and the population density of the bacteria only. However, when transfer rates decrease or when the microbial population and concomitant substrate consumption grow, mass transfer becomes limiting and linear growth is observed. Bioavailable substrate fluxes of poorly soluble compounds to the cells though can be controlled by changing interfacial area of the water-substrate interface [5].

3.1.1 Steps in the Protocol

1. Preparation of solid substrate: If reproducible growth is needed, solid substrates should be sieved to obtain defined size fractions.

2. Preparation of the cultivation bottles containing the desired growth medium and defined amounts of the solid substrates to obtain defined interfacial surface area (*see* **Notes** I and II).
3. Addition of the inoculum to the cultivation bottle and incubation at the desired temperature on a rotary shaker at 100–150 rpm.
4. Following microbial growth by, e.g. optical density measurements of the bacteria (*see* **Notes** III and IV).

3.1.2 Time Considerations

Time needed for cultivation depends on the microbial growth kinetics and substrate release rates. In case of sufficiently high release rates, exponential growth will be observed. If release rates are lower, linear growth will take place and thus significantly increase the cultivation time [4, 5].

3.2 Protocol 2: Cultivation Using a Polymer-Based Controlled Hydrocarbon Release System

In this protocol, excess quantity of a hydrocarbon with log K_{ow} ranging from 3.3 to 7.1 [14, 15] or of hydrocarbon mixtures is sorbed in an inert poly(dimethylsiloxane) silicone (PDMS) piece. The PDMS piece serves as a reservoir for controlled release of hydrocarbons to hydrocarbonoclastic [cf. Eq. (1)] and non-hydrocarbonoclastic organisms. In the latter case, release will lead to aqueous equilibrium partitioning concentrations in the range of the aqueous solubility down to zero, as can be calculated from a hydrocarbon's PDMS-medium partitioning constant ($K_{HC;PDMS:Medium} = c_{HC;PDMS}/c_{HC;Medium}$). The advantage of this protocol is that the hydrocarbon-containing PDMS pieces (i.e. the hydrocarbon-containing second phase) can be easily removed or added at any time during cultivation.

3.2.1 Steps in the Protocol

1. Preparation and loading of the PDMS hydrocarbon release system [16]:
   (a) In order to remove impurities of the PDMS manufacturing process, clean all PDMS pieces by immersing them overnight in a shaken flask containing ethyl acetate/water (1:10 (v/v)). Continue the cleaning process by consecutively immersing the PDMS three times in MeOH/water (1:15 (v/v)) for 24 h.
   (b) Prepare a saturated methanolic stock solution of the hydrocarbon and dilute it with MeOH to obtain the desired loading solution.
   (c) Expose dried PDMS pieces to the loading solution for at least 72 h to obtain thermodynamic partitioning equilibrium.
   (d) Discard the methanol suspension and rinse the PDMS pieces with a small volume of deionized water to remove residual methanol before usage.

(e) Quantify the target hydrocarbon in the PDMS pieces by chemical extraction with MeOH and subsequent chemical analysis (e.g. by GC–MS). *See* **Note** V.

(f) If needed, determine the hydrocarbon's PDMS-medium partitioning constant in order to calculate the initial equilibrium distribution of the hydrocarbon in the given system and/or determine the release rate for a given cultivation treatment to allow and adjust desired growth conditions. For these purposes, incubate the PDMS piece in desired medium to quantify the ratio between the concentrations of the hydrocarbon in the liquid phase to its concentration in the PDMS at equilibrium.

2. Cultivation:

(a) Transfer the loaded PDMS pieces to the cultivation flasks containing the desired medium (e.g. mineral salt medium) and let the system equilibrate under gentle shaking for >10 h in order to establish the calculated initial exposure concentration. *See* **Note** VI.

(b) Add the inoculum to the cultivation flask and incubate at the desired temperature on a rotary shaker and follow microbial growth by quantification of the cell number. *See* **Note** VII.

3.2.2 Time Considerations

To set up a PDMS-based hydrocarbon release system of defined release kinetics, three or more weeks should be planned. The cultivation itself depends on the growth kinetics of the organisms and the hydrocarbon release rates from the loaded PDMS polymer.

3.3 Protocol 3: Cultivation on Potentially Toxic Substrates

This protocol exemplifies a cultivation technique for organohalide-respiring bacteria using a biocompatible but non-biodegradable NAPL (hexadecane) to control the mass transfer rates and concomitant concentrations of growth substrates (tetra- and trichloroethenes) which are toxic at too elevated aqueous concentrations in batch cultures [17]. Adaptations of the protocol described below have also been used for the enrichment of bacteria dechlorinating trichlorobenzene and other chlorinated compounds [18].

3.3.1 Steps in the Protocol

1. Determination of the partitioning of target compound between the hexadecane and water phase:

(a) Create a hexadecane solution of known chloroethene concentration, make a dilution series of the same, and place it in closed bottles of known headspace and water volume.

(b) Allow for equilibration for 6 h at room temperature, withdraw a defined volume of the water phase with a glass

syringe, and quantify the target chloroethene by gas chromatography (e.g. after extraction with hexane).

2. Preparation of the cultivation bottles with the desired medium (e.g. a medium low in chloride) [18] and gas phase (N_2/CO_2 or H_2/CO_2). *See* also **Note** VIII.
3. Preparation of sterile hexadecane solutions of defined chloroethene concentrations:
   (a) Add a defined volume of hexadecane to a bottle and close with Viton® stopper.
   (b) Replace air in the gas phase by nitrogen gas (N_2).
   (c) Sterilize the hexadecane in an autoclave.
   (d) Add defined amounts of chloroethene to the hexadecane with a syringe through a sterile 0.2 μm Teflon filter.
4. Add the inoculum to the cultivation bottle with addition of a defined volume of chloroethene in hexadecane solution (*see* **Notes** IX and X).
5. Incubate at the desired temperature statically or on a rotary shaker at low speed (<100 rpm; *see* **Note** XI) and analyse dechlorination activity by analysing chloride production and chloroethenes in the gas phase using standard calibration curves.

3.3.2 Time Considerations

Enrichment of dechlorinating activity is not a rapid process (in particular if dechlorination beyond dichloroethene is strived for), and weekly or biweekly sampling is recommended for ca. 3 months. If no activity has been detected within this period, dechlorinating activity is unlikely to take place at a later stage.

3.4 Protocol 4: Cultivation on Substrates Provided via the Gas Phase

This protocol describes the enrichment and cultivation of aerobic dichlorobenzene-degrading bacteria using volatile substrates provided via the gas phase to avoid excessive toxicity [19–21].

3.4.1 Steps in the Protocol

1. Set-up of chemostat for enrichment:
   (a) Prepare the chemostat using, for example, the mineral salts medium described by Dorn et al. [22] *See* **Note** XII.
   (b) Install an aeration system allowing aeration at rates between 3 and 15 $l_{air}\, l_{medium}^{-1}\, h^{-1}$.
   (c) Install a system where the air passes through the liquid volatile substrate or a column containing substrate crystals. *See* **Note** XIII.
   (d) Inoculate the system and start with a low substrate concentration and increase stepwise (*see* **Note** XIII).

2. Cultivation on agar plates:
   (a) Prepare agar plates with mineral salts medium in Petri dishes.
   (b) Spread chemostat samples on agar and incubate plates in a desiccator containing the substrate in a tube allowing evaporation. *See* **Note** XIV.
   (c) Incubate agar plates at desired temperature.
   (d) Transfer colonies on control plates and incubate without carbon source supplied via gas phase.
3. Cultivation in liquid medium batch cultures:
   (a) Prepare mineral salts medium and add to baffled Erlenmeyer flasks, approximately 8% of total flask volume, and autoclave.
   (b) Inoculate and close air tight with screw cap equipped with inert solvent-resistant seal.
   (c) Add volatile substrate by syringe through septum of sidearm.
   (d) Incubate on rotary shaker at 150–400 rpm

4 Notes

I. Growth rates depend on the total number of solid particles (or liquid droplets) and their surface areas [5]. However, with high amounts of solids (>1 gL^{-1}), abiotic release rates may not be linearly proportional to the solids' surface area probably due to microscopic clumping effects of the solids [5].

II. In case of liquid compounds, the interfacial area between medium and NAPL can be influenced by the shaking speed and amplitude of the gyratory shaker and the size and form of the glassware used.

III. Microbial growth rates at low bioavailability conditions normally are not directly proportional to the release fluxes. This is due to the fact that microorganisms use a given fraction of the substrate released from the nonaqueous phase for their maintenance, which reflects the substrate consumption for nongrowth processes such as endogenous metabolism and compensation for cell decay [23]. Growth at mass-transfer-limited conditions may even stop at low optical densities when substrate release flux does not any longer cope with the culture's maintenance requirements. Growth will recover when release flux is increased, e.g. adding additional solid substrates to the culture medium.

IV. If the solid substrates interfere with OD measurements, i.e. do not settle fast enough, biomass may be analysed by protein quantification.

V. Please be aware that the PDMS piece will swell. Swelling during cleaning, loading, and extraction protocols is dependent on the properties of the polymeric phase and the solvent used. The application of methanol has the advantages that it causes limited swelling, can be completely removed with water, and prevents frangibility of the polymer [24].

VI. Aqueous steady-state hydrocarbon concentrations are normally established within a few hours [25].

VII. Delivery fluxes of the hydrocarbon from the PDMS should be balanced with the growth rates of the organisms. Therefore the release of the hydrocarbon into the aqueous phase depends on the size and geometric shape of the PDMS material and the mass transfer velocity of hydrocarbon from the silicon into the aqueous phase. Sophisticated dimensioning of the system and an appropriate shaking velocity improve the release kinetics of the hydrocarbon to allow optimal growth conditions [16, 26].

VIII. If hydrogen is used as energy and electron source, dechlorinating bacteria are enriched best in the presence of acetate as carbon source. In case other organics (such as ethanol, propionate, and butyrate or mixtures of them) are chosen, they should be added semi-continuously (e.g. weekly) in order not to exceed concentrations of 1–2 mM.

IX. Inject the hexadecane slowly avoiding the formation of water-hexadecane emulsions that can have adverse effects on bacterial growth.

X. Calculate the amount to add in order to avoid maximal chloride concentration in the medium of >20–30 mM assuming quantitative dechlorination of the target compound. This avoids excessive acidification of the medium as reductive dechlorination produces hydrochloric acid and reduces the buffering of the medium.

XI. Vigorous mixing of the water and organic solvent phase can inhibit bacterial growth. In addition, incubation temperature depends on the melting point of the organic solvent used. For hexadecane, incubations below 15°C are not possible.

XII. The pumps should allow a dilution rate between 0.005 and 0.1 h^{-1}. pH and temperature control units help to avoid acidification and excessive temperature changes.

XIII. Preferable is a system to control the gas phase concentration, which should be followed by gas chromatography. For the enrichment of the *o*-DCB-degrading *Pseudomonas* sp., for

example, the gas-phase *o*-DCB concentrations were raised from initially 0.3 to 3 mg l^{-1} over a period of 2 months [21].

XIV. If needed, dissolution of the substrate into a non-volatile NAPL will reduce its evaporation fluxes and reduce its concentration in the desiccator's gas phase.

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Protocol for Isolation, Screening, and Cultivation of Asphaltene-Degrading Microorganism

Seyed Morteza Zamir, Tina Tavassoli, and Seyed Abbas Shojaosadati

Abstract

Precipitation of heavy hydrocarbons, especially wax and asphaltenes, is a major problem in oil-related industries. The presence of asphaltenes results in higher viscosity, emulsion formation, coke production, and consequently clogging of pipelines. These compounds contain metals such as iron, nickel, and vanadium and hence are poisonous for catalysts in refinery processes. This has led to a strong incentive to find microorganisms which are able to cleave asphaltene compounds and thus promote facile exploitation of these resources.

Keywords: Asphaltenes, Biodegradation, Isolation, Screening, Cultivation

1 Introduction

Petroleum consists of hydrocarbons with various molecular weights. At high temperature and pressure in oil reservoirs, heavy compounds are in a thermodynamic equilibrium with other components such as dissolved gas in oil which helps stabilizing these heavy compounds in the solution. After oil extraction and change in temperature and pressure and separation of oil-associated gases, the thermodynamic equilibrium of petroleum components changes and heavy compounds begin to deposit [1].

1.1 Asphaltenes

In general, asphaltenes are molecules that can be found in crude oil, which are insoluble in normal light alkanes and soluble in benzene and toluene. Asphaltenes are often introduced as the heaviest and the most polar oil compounds with amorphous molecules. Asphaltenes do not melt; rather, they degrade at temperatures higher than 300–400°C and produce carbon and volatile products [2].

1.1.1 Chemical Structure

Asphaltenes consist of aromatic compounds with alkane chains. The most abundant element is carbon with composition of 80–88% (w/w), followed by hydrogen 7.5–11% (w/w), and sulfur

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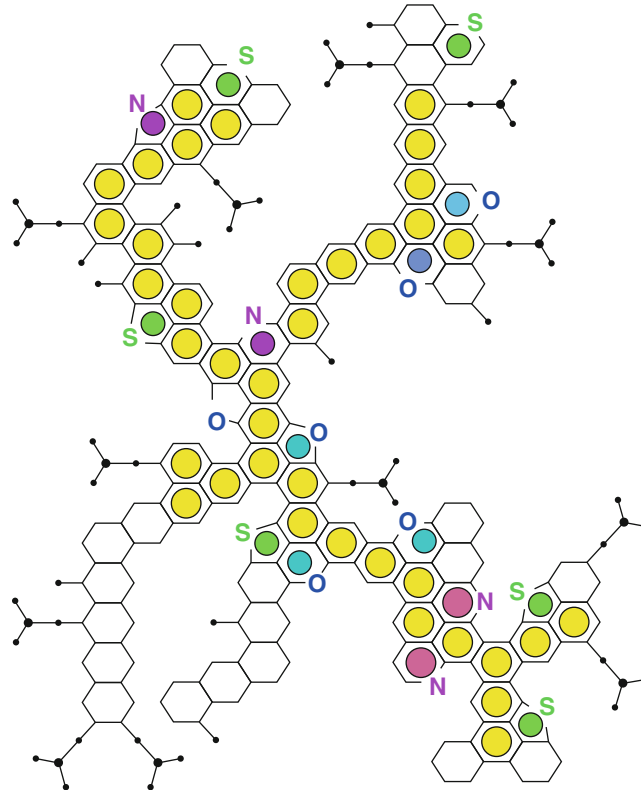

Fig. 1 Exemplar molecular structure of asphaltenes extracted from crude oil [3]

0.3–10.3% (w/w) [2]. The elemental composition in precipitations of asphaltenes differs according to the oil reservoirs. The presence of sulfur compounds in asphaltenes causes corrosion and results in numerous other problems in facilities. Asphaltenes contain also oxygen 0.3–4.8%, nitrogen 0.6–3.3%, and the metals iron, nickel, and vanadium 0.3–0.5%. They are the heaviest and the most polar components of crude oil with an estimated molecular weight of 600–2,000,000 g/mol. The typical chemical formula for asphaltenes is $C_{74}H_{81}NS_2O$ (Fig. 1). Isolated asphaltene is blackish-brown colored. Changes in temperature, pressure, and oil density result in precipitation of asphaltenes in different stages of the oil refining process. The main problem in oil recovery and refining is related to deposition and agglomeration of asphaltenes, because its particles bind to each other and agglomerate in the presence of paraffin hydrocarbons. Accurate measurement of molecular weight and agglomerate size is difficult due to unknown agglomeration mechanism. The molecular weight of agglomerates ranges from a few hundred to millions of grams per mole.

A number of chemists have tried to describe asphaltene's molecular structure and to identify its constituent elements and hence have modeled its molecular structure through physical and chemical methods [4].

Asphaltene precipitation is one of the best known and most important problems in the oil industry, particularly in extraction, resulting in various problems such as augmented extraction costs and reduced oil recovery from reservoirs. Blockage of porous medium in oil formations, well openings, and processing facilities is a serious problem caused by asphaltene-rich oils. Therefore, modeling of asphaltene precipitation is important, since injection and production systems can be designed so that asphaltene precipitation is minimized or negated. However, most proposed models lack high precision and cannot be applied in all cases of asphaltene deposition, because the deposition has unknown compounds and no similar molecular structure can be considered for them; in essence, the structure of these compounds varies in different reservoirs. Thus, studying a method that can remove asphaltene precipitation is of special importance.

1.1.2 Common Ways of Coping with Precipitation

Considering all the problems mentioned resulting from deposition in various equipment, pipelines, etc., precipitation should be avoided as much as possible. Since precipitation cannot be completely halted, necessary measures should be taken in order to remove deposition. In general, the following methods are used to resolve the precipitation-induced problems: mechanical, thermal, chemical (solvent application), electromagnetic, chemical surfactants, crystal modifiers, ultrasound, and biological degradation [5–7]. These methods have been used so far to remove precipitation, including the common method of organic aromatic solvents such as toluene and hot xylene which exert numerous environmental problems due to their carcinogenesis. In addition, mechanical and thermal methods are used in this field and are relatively expensive. These disadvantages, beside environmental restrictions, have led to numerous attempts to apply environmentally friendly methods to remove oil pollution and asphaltene deposition. In this regard, utilization of microorganisms that are able to consume heavy hydrocarbons as their carbon source is potentially a promising method.

1.2 Biodegradation of Hydrocarbons

Light hydrocarbons or compounds with medium molecular weight are degraded more readily than heavier compounds. In addition, degradation of paraffinic compounds is generally easier than aromatics [8].

Despite little information on microbial degradation of asphaltenes, based on previous experiences from degradation of lighter compounds, possible cleavage or biodegradation of asphaltenes can be estimated. Figure 2 depicts these positions.

Mass transfer rate of hydrophobic molecules of asphaltenes to biocatalysts is low in the aqueous phase [9]. Furthermore, the characteristics of the bacteria are crucial. Usually, oil-polluted soil with long-term contact with oil is a good source for isolation of

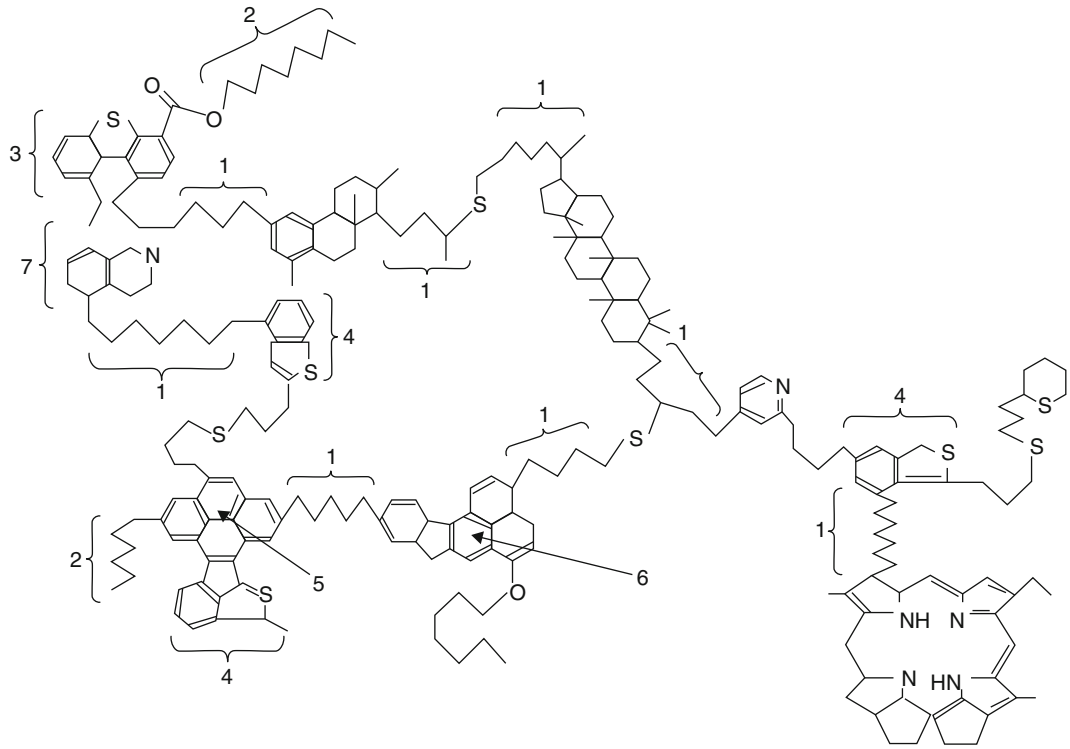

Fig. 2 Representation of intermolecular bonds of asphaltenes with the possibility for cleavage: *1* photooxidation, *2* p-oxidation, *3* dibenzothiophene pathway, *4* dibenzothiophene-like pathways, *5* pyrene pathways, *6* benzo(a)pyrene-like pathways, and *7* carbazole degradation-like pathways [8]

bacteria for asphaltene biodegradation. Few studies have been carried out, and the isolated bacteria were mainly *Bacillus*, *Brevibacillus*, *Staphylococcus*, and *Pseudomonas* species [10, 11]. Moreover, there are some reports about the ability of some fungi to degrade or even partially mineralize asphaltenes as their sole carbon and energy sources via the production of extracellular enzymes and surfactants [12]. Fungi also have hyphae that can attach or move through the structure of asphaltenes, facilitating degradation.

Application of biodegradation methods in removing oil pollutants and precipitations is generally more environmentally friendly and economical than physicochemical methods. In spite of the advantages of biological methods, they have some limitations such as slow rate of reaction in some cases and maintaining sterile conditions in the case of working with pure cultures.

2 Materials

2.1 Enrichment Culture Media

- Nutrient broth medium: This medium is used for the enrichment of various types of bacteria in soil and crude oil. It can be purchased from Fluka.

Table 1
Chemical composition of growth medium (ISO 9439)

Compounds[a]	Concentration (g/L)
KH_2PO_4	0.08
K_2HPO_4	0.21
$Na_2HPO_4 \cdot 2H_2O$	0.33
NH_4Cl	0.005
$MgSO_4 \cdot 7H_2O$	0.0225
$CaCl_2$	0.0275
$FeCl_3 \cdot 6H_2O$	0.025

[a]All chemicals used from Merck (**Note 1**)

2.2 Isolation Culture Media

- Nutrient agar medium: This medium is used for the isolation, purification, and storage of bacteria. It can be purchased from Fluka.

2.3 Growth Media

- Special culture medium: This medium is especially used for the growth of bacteria using asphaltenes as their sole carbon and energy source. Since the only carbon source in this culture medium is asphaltenes, only those bacteria using asphaltenes as their carbon source will be able to grow. This medium is also called ISO 9439 [10]. Table 1 presents the composition of this culture medium.
- *n*-Heptane: Analytical grade as the standard organic solvent for the extraction of asphaltenes from Sigma-Aldrich (**Note 1**).
- Samples of crude oil and petroleum-contaminated soil from local area.

3 Methods

The protocol described below provides the procedure for isolation and screening of microorganisms capable of biodegrading asphaltenes. Each experiment must be performed at least in duplicate. Provide two samples for each test and report the average results of two experiments. During each phase of the experiments, observation of microbial cultures under a microscope is recommended to distinguish the different types of microbes.

Also, it should be pointed out that asphaltene biodegradation must be proved based on structural analysis – i.e., using techniques such as Fourier transform infrared (FTIR) and elemental analysis (CHN) – and not just by gravimetric method. The reason for this is

that the probable production of extracellular polymers or biosurfactants during biodegradation may liberate trapped hydrocarbons in the asphaltic matrix and make a significant error in weight measurement [12].

FTIR is a method to determine chemical bonds and molecular structure of organic and inorganic materials. Each type of chemical bond absorbs infrared rays at a specific frequency, which is the characteristics of that bond. Regarding the amount of absorbance and transmission of the infrared in each frequency, the obtained inverse peaks show the intensity of each bond in the sample.

3.1 Asphaltene Precipitation

Asphaltenes must be extracted from crude oil to be prepared for biodegradation. This process is very important. If extraction or precipitation is not performed carefully, some light fractions of oil such as aromatic components may remain in the asphaltene structure. Therefore, some microorganisms may utilize them as sole carbon/energy source instead of degrading asphaltenes. In this case, target microorganisms – i.e., asphaltene degraders – may be lost during steps of isolation and screening.

1. The asphaltene is precipitated from the crude oil sample by addition of 20 mL *n*-heptane to 1 mL crude oil (ideally heavy crude oil) and using magnetic agitation for 18 h.
2. Filter the suspension using Whatman No. 42 filter paper in a vacuum filtration system.
3. The precipitated asphaltenes on the filter should be dried at 70°C for 24 h and kept at room temperature in a desiccator. The remaining asphaltenes can be used in different experiments (**Note 2**).

3.2 Enrichment of Microbial Culture

First, it is necessary to grow the microorganisms of polluted soil samples in the nutrient broth medium to increase their abundance.

1. Add 2 mL of crude oil sample and/or 2 g of polluted soil (**Note 3**) aseptically to 98 mL of sterile (**Note 4**) nutrient broth medium in a 500 mL Erlenmeyer flask.
2. The samples should be incubated at 28°C (**Note 5**) with shaking at 200 rpm for 7 days.
3. After 7 days, transfer 2 mL of the incubated culture to 100 mL fresh nutrient broth and incubate it again at the same condition. At this stage, usually visible growth of microorganisms can be seen inside the flasks.

3.3 Isolation of the Microorganisms

The isolation and cultivation of microorganisms capable of utilizing asphaltenes as their sole carbon or energy source is discussed.

1. Sterilize (**Note 4**) a mixture of 0.5 g asphaltenes together with 50 mL of ISO 9439 medium or other minimal medium

reflecting the desired ionic composition and nutrient concentration. Trace elements and vitamins may be added (*see* other chapters).

2. Add 2 mL of enriched culture (*see* Subheading 3.2) to the flask (**Note 6**).
3. Adjust pH to about 7.5 by appropriate addition of HCl (1 N) or NaOH (1 N).
4. The samples should be incubated at 28°C and 200 rpm for 30 days.

3.4 Screening Procedure

As there is likely to be simultaneous growth of different microorganisms in enrichment (Subheading 3.3), the members of the microbial consortium must be separated from each other by serial dilution in order to obtain pure cultures.

1. Prepare seven sterile nutrient agar plates (**Note 7**).
2. Prepare seven sterile test tubes – with a volume of approximately 10 mL – containing 0.9 mL sterile distilled water (**Note 8**).
3. Add 0.1 mL of enriched culture to the first tube and shake it vigorously for 1 min using a vortex mixer.
4. While the sample is still in swirling, add 0.1 mL from the first tube to the second and repeat.
5. Keep on diluting until all tubes have been inoculated by the culture.
6. Add 100 μL of each diluted enrichment (1–7) to the surface of nutrient agar plates and move them slowly for 1 min on the table for uniform dispersion of liquid on the surface of agar.
7. Keep the plates in a static incubator at 30°C for 24 h or until visible colonies can be observed.
8. Select those plates with separate colonies and cultivate all colonies with different morphologies separately again on new nutrient agar plates.
9. Repeat **steps 7** and **8** until a visible pure colony can be seen (**Note 9**).

3.5 Cultivation of Asphaltene-Degrading Microorganisms

To ensure that screened microorganisms are capable of utilizing asphaltenes as the sole carbon source and to quantify the biodegrading ability of each culture, particular experiments for the biodegradation of asphaltenes must be carried out. For each pure culture obtained, perform the following experiment at least in duplicate.

1. Add 0.5 g asphaltenes to 50 mL ISO 9439 medium in a 100 mL Erlenmeyer flask.
2. Prepare another flask containing only 50 mL ISO 9439 medium.

3. Sterilize both flasks.
4. Collect enough culture from the plate (Subheading 3.4) with a sterilized loop and transfer it to the flasks. Now, there is one flask including asphaltenes as the sole carbon source, while the other has no carbon source. If the screening procedure is correctly performed, the microorganism will only grow in the flask containing asphaltenes (**Note 10**).
5. Incubate flasks at 28°C and 200 rpm for 60 days.
6. The biodegrading ability of each culture is evaluated using IP 143 method (**Note 2**). Add 1 mg asphaltenes from the incubated culture to 20 mL *n*-heptane and using a magnetic agitation for 18 h.
7. Filter the suspension using Whatman No. 42 filter paper in a vacuum filtration system.
8. The precipitated asphaltenes on the filter should be dried at 70°C for 24 h and kept at room temperature. The remaining asphaltenes can be analyzed by FTIR spectroscopy to investigate the variation of chemical bonds and molecular structure (**Note 11**). We use FTIR model Nexus 670, Thermo Nicolet Co, USA. Figure 3 presents a FTIR spectra for both untreated and biodegraded asphaltenes.
9. Send two samples of biodegraded and untreated asphaltenes for CHN elemental analysis (**Note 12**). We use the model of CHNORAPID, Germany. CHN analysis is based on complete combustion of dry samples to determine the forming ratio of carbon, hydrogen, and nitrogen of the samples. The H/C ratio must be higher in biodegraded sample than that of untreated sample.

4 Notes

1. In the list of materials, we provide the names of vendors we purchased the chemicals for the cultivation of asphaltene-degrading microorganisms. However, there are other companies that provide these chemicals. Take care of the chemicals' grade.
2. The process of asphaltenes extraction by *n*-heptane, vacuum filtration, and drying is called IP 143 (http://publishing.energyinst.org/publication/ip-standard-test-methods/ip-143-determination-of-asphaltenes-heptane-insolubles-in-crude-petroleum-and-petroleum-products).
3. If there are samples from oil and from soil wherein oil reservoir is located, it is recommended to add each of them individually to the flasks for microbial enrichment, because it is highly

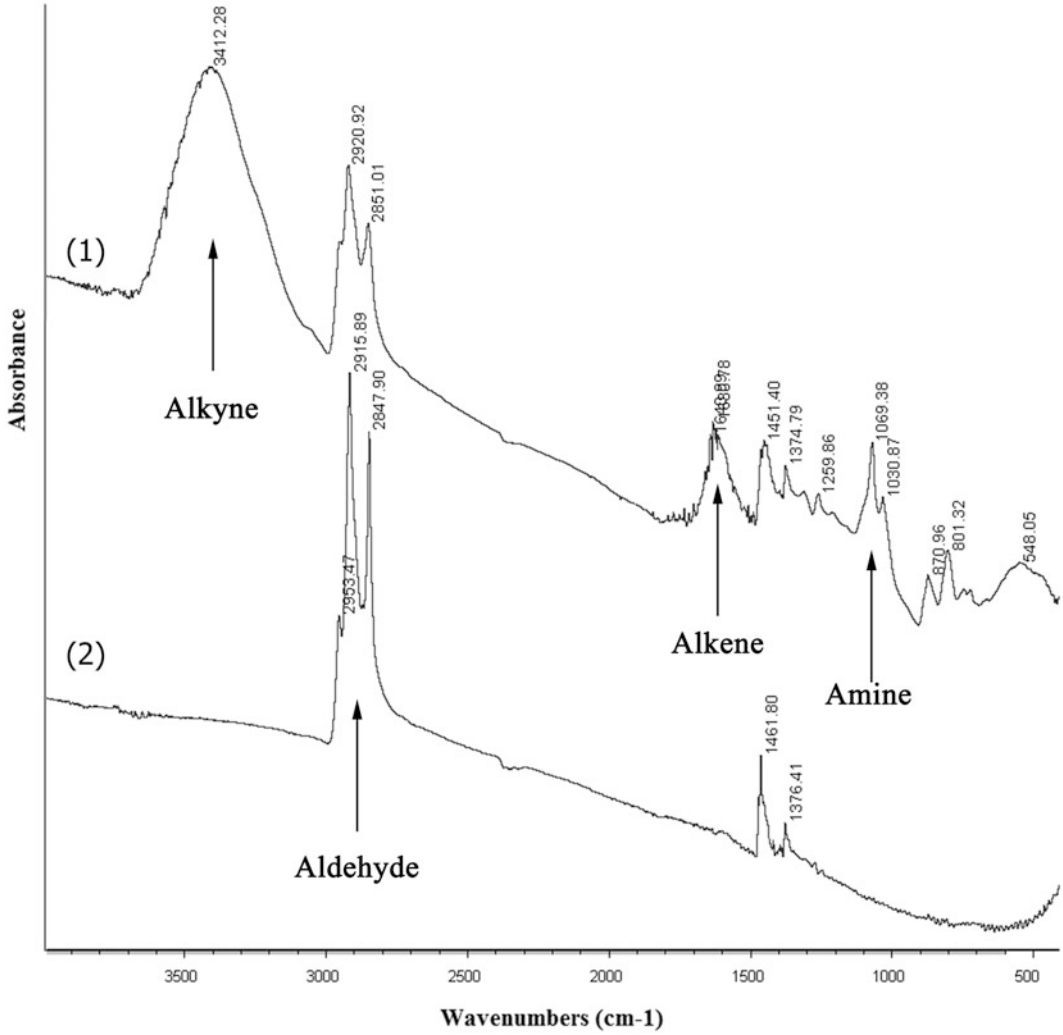

Fig. 3 FTIR analysis results (*1*) untreated asphaltenes and (*2*) biodegraded asphaltenes. The peak at 3,412 cm^{-1} in untreated sample belongs to C=C bonds which has been removed in the biodegraded sample. Also, the peaks at 1,650 and 1,070–1,030 cm^{-1} in untreated sample are related to C=C bonds and amine groups, respectively, which have been eliminated in the biodegraded sample. In contrast, aldehyde and methyl groups (wave numbers of 2,900–2,800 and 1,450–1,300 cm^{-1}, respectively) have not been degraded significantly [11]

probable that microorganisms capable of degrading asphaltenes can also be found in the environment of reservoir.

4. A standard sterilization process includes autoclaving at 121°C for 15 min. This time is sufficient for common medium sterilization. However, in case of soil sample sterilization, the required time may need to be increased to about 30 min, or tyndallization may be performed whereby the sample is autoclaved on 3 consecutive days.

5. The incubating temperature depends on the target microbes. A common mesophilic temperature for most bacterial and some fungal growth is 28°C. If extracellular enzymes production by white-rot fungi is needed, 37°C can be used. If the aim of the research is the thermophilic asphaltene biodegradation, temperatures over 50°C are suggested.
6. It is highly recommended to perform the inoculation process under a laminar flow hood.
7. Prepare 2–3% (w/v) nutrient agar by adding 2–3 g nutrient agar to 100 mL distilled water and autoclave it. Also, it should be pointed out that nutrient agar is given as an example. Microbes may be enriched with any suitable medium. Therefore, it is recommended to employ a range of different growth media.
8. The extent of dilution depends on microbial abundance in the samples. Seven-stage dilution is usually appropriate for this purpose. Nevertheless, prepare more test tubes and dilute the culture until separate colonies are formed.
9. In some cases, streaking colonies onto new agar plates is useful to obtain a pure culture. If no differences in shape or color could be seen in the streak, the colony is likely to be pure. If not, further dilution (Subheading 3.4) is needed.
10. Microorganisms usually grow as biofilm around the asphaltenes. To detach biofilm from asphaltenes, shake the flask vigorously by hand. For determination of biomass, use the dry cell weight method. In fact, the residual asphaltenes must be separated and washed from liquid medium carefully in a beaker. Then, centrifuge the whole liquid part at 5,000 rpm (~2,795 g) for 10 min. Dry the sediment at 70°C to reach a constant weight. The growth of the organisms is determined as dry weight.
11. Prepare a sample of untreated asphaltenes as blank sample, too. Consult with FTIR operator before preparing the samples. The operator may list some conditions that should be taken into account by researcher in terms of minimum weight/volume of samples, drying them in an oven, etc.
12. CHN analysis should be carried out according to the common method of ASTM D5291 (http://www.astm.org/Standards/D5291.htm). Consult with CHN analysis operator to prepare an appropriate sample. The operator may ask to dry sample under special condition, as usually humidity interferes the results from CHN analysis.

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Cultivation of Anaerobic Microorganisms with Hydrocarbons as Growth Substrates

F. Widdel

Abstract

Microorganisms utilizing hydrocarbons anaerobically as organic growth substrates are grown in defined anoxic media with sulfate, nitrate, or other electron acceptors. Cultivation of such microorganisms is technically often more elaborate than that of other anaerobes. Special technical measures include, for instance, dilution of potentially toxic hydrocarbons in an inert carrier phase and precautions to avoid diffusion of traces of oxygen through stoppers during long incubations. Furthermore, contact of liquid hydrocarbons with stoppers should be avoided.

Keywords: Anaerobes, Cultivation, Gases, Hydrocarbons, Solubility

1 Introduction

1.1 Principal Considerations

Cultivation of microorganisms with hydrocarbons as growth substrates under anoxic conditions is more demanding than cultivation of conventional anaerobes. Four general points must be taken into consideration:

1. Anaerobic growth with hydrocarbons is very slow. Whereas doubling times of aerobic hydrocarbon degraders are in the range of several hours, doubling times of anaerobic hydrocarbon degraders are in the order of days to several weeks (the latter in the case of anaerobic methane oxidizers). Most experiments depending on growth therefore include long "waiting times." Also cell densities are much lower than those of aerobes.
2. Maintenance of strictly anoxic conditions must be ensured. Especially during the long incubation times, oxygen can diffuse through stoppers and inhibit growth of anaerobes. On the other hand, small amounts of O_2 may allow hydrocarbon activation and lead to partly oxygenated products that can be degraded further. Such "pseudoanaerobic" growth with

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hydrocarbons may occur especially in enrichment cultures or in cultures of denitrifiers, which are facultative aerobes.

3. Liquid and solid hydrocarbons are mostly very poorly water soluble (see Wilkes and Schwarzbauer in this Volume), and their availability for the bacteria in the medium may be strongly limited. So far, known, anaerobic hydrocarbon degraders do not produce surfactants to increase hydrocarbon availability in the aqueous phase. Hence, if possible, the hydrocarbon-water interface should be maximized so as to increase diffusion and/or the surface area for contact with cells. On the other hand, hydrocarbons strongly adsorb to stoppers. This may lead to losses of hydrocarbons, which is critical in quantitative growth experiments, and to deterioration of stoppers. Contact between hydrocarbons and stoppers must therefore be kept as minute as possible.
4. Most pure liquid hydrocarbons with noticeable vapor pressure are toxic if added as pure substances (viz., with chemical activity = 1), in particular due to their interaction with lipid membranes. Such hydrocarbons must be provided at lower chemical activity. This can be achieved by their dissolution in hydrophobic inert carriers such as nonvolatile branched-chain alkanes.

1.2 Technical and General Remarks About Preparing Anoxic Media and Working Under Anoxic Conditions

Techniques involving O_2-free N_2 or mixtures of N_2 and CO_2 to prevent entrance of air during handling of culture flasks and tubes are compulsory. An anoxic chamber is useful but not obligatory, unless enzymes are to be purified from anaerobic hydrocarbon degraders. There are convenient gassing techniques, such as the use of the classical cotton-filled Hungate syringe with a bent needle, which allow work with anaerobes at a normal laboratory bench. Details of these techniques have been published [1–3] and are not repeated here.

The use of defined, transparent media that do not contain organic nutrients other than the hydrocarbon substrate is recommended. An exception is the addition of ascorbate as a mild, compatible reductant (scavenger of traces of oxygen) to pure cultures of denitrifiers if this does not serve as a growth substrate (which is usually the case). The medium is prepared in subsequent steps rather than in one batch. The heat-stable nonvolatile major mineral salts are dissolved and autoclaved. Components that undergo chemical changes (e.g., vitamins) or volatilization (e.g., hydrogen sulfide) in the heat are added from separately sterilized stock solutions after autoclaving and cooling of the major mineral salts solution.

Description of the following media is given for sulfate- and nitrate-reducing microorganisms, the most frequently studied anaerobic hydrocarbon degraders, and for methanogenic cultures which have more recently become of interest. Anaerobic hydrocarbon degradation coupled to sulfate reduction is presumably

quantitatively more important in nature (e.g., in marine sediments, hydrocarbon seeps, and oil fields) than their degradation coupled to nitrate. However, denitrifying anaerobic hydrocarbon degraders usually grow much faster, have higher growth yields, and are therefore more convenient for cultivation in the laboratory, for instance, for enzymatic studies.

1.3 Materials (Concentrated Aqueous Stock Solutions)

1.3.1 Trace-Element Mixture A (Chelated and Non-chelated Forms)

A non-chelated trace-element solution has been frequently used for sulfate-reducing bacteria. The included acid prevents formation of ferric precipitates during storage. A chelated solution is common for nitrate-reducing bacteria (Table 1).

Use only fresh, greenish crystals of $FeSO_4 \cdot 7H_2O$; brownish grains indicate weathering and oxidation. Autoclave, preferentially anoxically under N_2.

1.3.2 Trace Element Mixture B

Distilled H_2O	1,000 ml
NaOH	400 mg
$Na_2SeO_3 \cdot 5H_2O$	6 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
$Na_2WO_4 \cdot 2H_2O$	8 mg

Autoclave. Some colorless flocs that may be formed by reaction of the alkaline solution with some bottle glasses are harmless.

Table 1
Trace-element mixture A

	Non-chelated solution	Chelated solution
Distilled H_2O	987 ml	1,000 ml
HCl (25% = 7.7 M)	13 ml	None
EDTA, Na_2 salt	None	5,200 mg
H_3BO_3	10 mg	10 mg
$MnCl_2 \cdot 4H_2O$	5 mg	5 mg
$FeSO_4 \cdot 7H_2O$	2,100 mg	2,100 mg
$CoCl_2 \cdot 6H_2O$	190 mg	190 mg
$NiCl_2 \cdot 6H_2O$	24 mg	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg	10 mg
$ZnSO_4 \cdot 7H_2O$	144 mg	144 mg
pH adjustment	none	To 6.0 (with 1.0 M NaOH)

1.3.3 Bicarbonate Solution (1.0 M)

Dissolve 84 g $NaHCO_3$ in distilled water to a final volume of 1,000 ml. Appropriate portions may be prepared that can be used in full for medium batches (e.g., for 30 ml for 1 l, 60 ml for 2 l; see below). Saturate the solution with CO_2 by shaking in a stoppered bottle under a head space of CO_2. Autoclave in closed tubes or bottles with fixed stoppers (butyl rubber or Viton) under a head space of CO_2 (2:¼ of total volume).

1.3.4 Vitamin Mixture

NaH_2PO_4 + Na_2HPO_4 (10 mM total P; pH 7.1)	100 ml
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
D(+)-Pantothenic acid, Ca-salt	5 mg
Pyridoxine dihydrochloride	15 mg

Filter-sterilize (pore size, 0.2 μm) and store in the dark (preferentially in brown glass bottles) at 4°C.

1.3.5 Thiamine Solution

H_3PO_4+NaH_2PO_4 (10 mM total P; pH 3.4)	100 ml
Thiamine Cl·HCl	10 mg

Filter-sterilize (pore size, 0.2 μm) and store in the dark (preferentially in brown glass bottles) at 4°C.

1.3.6 Vitamin B_{12} Solution

Distilled H_2O	100 ml
Cyanocobalamin	5 mg

Filter-sterilize (pore size, 0.2 μm) and store in the dark (preferentially in brown glass bottles) at 4°C.

1.3.7 Sodium Sulfide Solution (0.2 M)

Take a few crystals that are not too small (ideally a single big crystal) of about 10 g $Na_2S \cdot 9H_2O$ from the storage jar; determine the exact weight, and add to a calibrated cylinder (capacity 250 ml). Use only colorless, clear crystals of sodium sulfide. In contact with air, stocks of sodium sulfide in commercial jars are easily oxidized to sodium thiosulfate and other salts. Large crystals with opaque or milky surface layers (oxidation products) can be briefly washed by rinsing with distilled water on a plastic sieve. Determine the weight after rinsing and drip-off of water. Add H_2O so as to adjust the solution to 48 g $Na_2S \cdot 9H_2O$ per liter (0.2 M). Dissolve the sodium sulfide by stirring under an N_2 atmosphere (close cylinder with stopper). Aliquot and autoclave the solution in closed tubes or bottles with fixed stoppers (butyl rubber or Viton) under a head space of N_2 (more than 25% of total volume).

1.3.8 Sodium Ascorbate Solution (0.5 M)

Distilled H_2O	40 ml
Ascorbic acid	9 g
NaOH (1.0 M), add slowly	40 ml

Add the NaOH solution slowly while stirring and cooling in an ice water bath, preferentially in a device that allows gassing with N_2 to avoid access of air. Add further NaOH dropwise until the pH is 8–9. Dilute with distilled H_2O to a final volume of 100 ml. Filter-sterilize and store anoxically under a head space of N_2 in the dark at 4°C.

2 Methods

2.1 Preparation of Media

Depending on the physiological type of microorganisms and the salinity of the original source, one of the following basal mineral media should be prepared. "F" (freshwater medium) is for microorganisms from freshwater habitats. "S" (simple saltwater medium) can be used for marine microorganisms that do not require magnesium and calcium ion concentrations as high as in natural seawater; the advantage of the relatively low concentration of these ions (in comparison to those in seawater) is that the pH can be increased without significant formation of precipitates. "M" (full marine medium) is used for marine microorganisms with unknown salt demands or which require high magnesium and calcium ion concentrations (as in natural seawater); a certain disadvantage of this medium is the formation of significant precipitates with increasing pH (Table 2).

Prepare media in special flasks with tubes for anoxic sterile gassing and a closable outlet that allows distribution of the complete medium to smaller cultivation tubes or bottles; devices have been described [3]. Dissolve the salts one after the other (simultaneous addition may lead to big precipitates) and autoclave. Cool the solution under an N_2–CO_2 mixture to prevent redissolution of oxygen. Then, add the other components from sterile stock solutions as indicated.

Do not use the sodium ascorbate solution for enrichment cultures. Adjust the pH to 7 with sterile 1 M Na_2CO_3 or 1 M HCl (autoclaved in closed bottle under gas head space) solution. Distribute the completed medium in culture tubes and bottles, and store anoxically under a head space of an N_2–CO_2 mixture. Add the hydrocarbon of interest individually to each tube or bottle as described in the following section.

Table 2
Defined media with different salinity

	Sulfate reducing			Nitrate reducing			Methanogenic		
	F	S	M	F	S	M	F	S	M
Dissolve one after the other with stirring per liter of dist. H_2O (amounts in g)									
NaCl	–	20.0	26.0	–	20.0	26.0	0.5	20.0	26.0
$MgCl_2 \cdot 6H_2O$	0.5	3.0	10.0	0.5 g	3.0	10.0	0.5	3.0	10.0
$CaCl_2 \cdot 2H_2O$	0.1	0.15	1.4	0.1 g	0.15	1.4	0.1	0.15	1.4
NH_4Cl	0.3			0.3			0.3		
KH_2PO_{4a}	0.2			0.2			0.2		
KCl	0.5			0.5			0.5		
Na_2SO_4	4.0			0.05[b]			(0.05)[c]		
After autoclaving and anoxic cooling, add per liter (amounts in ml)									
Trace elements A	1.0[d] (non-chelated)			1.0[d] (chelated)			1.0[d] (non-chelated)		
Trace elements B	1.0[d]			1.0[d]			1.0[d]		
$NaHCO_3$-soln. (1.0 M)	30.0			30.0			30.0		
Vitamin mixture	1.0			1.0			1.0		
Thiamine soln.	1.0			1.0			1.0		
B_{12}-soln.	1.0			1.0			1.0		
N_2S-soln. (0.2 M)	5.0			–			5.0		
Na-ascorbate-soln. (0.5 M)	–			3.0 (for pure cultures)[e]			–		
$NaNO_3$-soln. (1.0 M)	–			4.0–10.0			–		
pH, adjusted with 1.0 HCl	Usually 6.9–7.1								

[a]For full marine medium (M) preferentially added from sterile KH_2PO_4 stock solution (0.5 M) after autoclaving and cooling of the other salts
[b]Sulfur source for cell synthesis
[c]May not be needed as a sulfur source, because sulfide will be added
[d]A few anaerobic cultures may be stimulated by higher amounts, e.g., 3 ml l^{-1} (to be tested with each solution)
[e]Not applicable in enrichment cultures, because ascorbate-degrading bacteria will be soon selected
F freshwater; *S* simple saltwater; *M* full marine for cultivation of anaerobes with hydrocarbons

2.2 Addition of Hydrocarbons

2.2.1 Gaseous Hydrocarbons

Gaseous hydrocarbons are obtained from steel bottles via gauges. Aseptic addition is guaranteed by passing the hydrocarbon gas through a sterile cotton or a membrane filter. The gaseous hydrocarbons may be injected through stoppers into the culture head space by means of syringes with hypodermic needles. The syringes should be pre-flushed anoxically. The added amount is obvious from the added volume (at 25°C, a volume of 24 ml of the pure gas at ambient pressure [101 kPa] is approx. 1 mmol).

Wastage of hydrocarbon gases and an open gas stream (as in the case of gassing with N_2) can be avoided by using a septum device for filling of syringes (Fig. 1).

The application of gaseous hydrocarbons with high overpressure (with the exception of methane) is usually not necessary. In the case of methane, an increased pressure may stimulate anaerobic methane-oxidizing communities. A safe device has been described that allows application of high pressure to methane in glass tubes (Fig. 2; [4]).

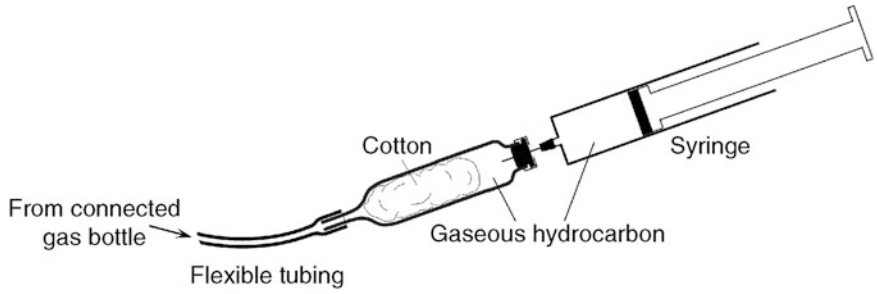

Fig. 1 Sparing filling of a syringe with gaseous hydrocarbons without "open" flushing via a special glass tube with connection to a bottle, sterile cotton, and a rubber septum. The syringe is initially flushed with N_2; avoid suction of air. For aseptic withdrawal, the rubber stopper may be sterilized with ethanol (allow to dry)

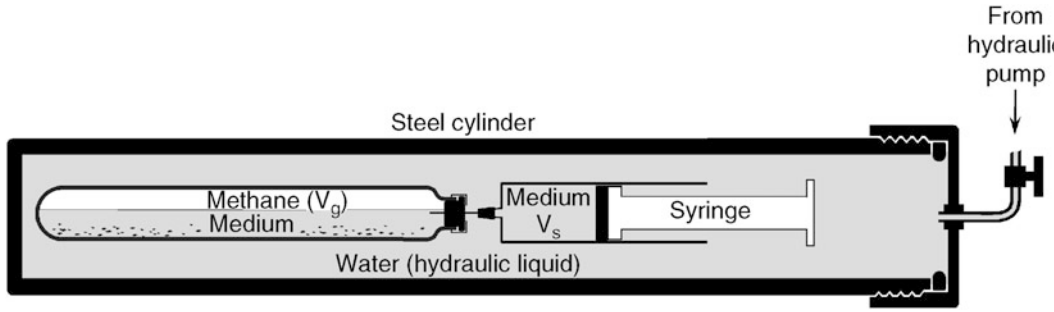

Fig. 2 Anaerobic incubation of a culture with methane at high pressure (high concentration of dissolved methane). The culture is prepared with a head space of methane. Additional medium is contained in the syringe. Upon pressurization, this medium is forced into the culture tube and the methane dissolved under the elevated pressure. The gas volume in the culture tube must be smaller than the volume in the syringe ($V_g < V_s$). In this way, a residual gas volume and implosion of the culture tube are avoided when the syringe is emptied by application of hydraulic pressure. According to Nauhaus et al. [4]

2.2.2 Liquid Hydrocarbons

Liquid hydrocarbons can be sterilized by filtration through solvent-resistant cellulose filters (pore size, 0.2 μm) or be autoclaved in tightly closed bottles with a head space (approx. ½ of bottle volume); in the case of volatile hydrocarbons, the weight should be controlled to reveal the tightness of the closure. For storage (as well as for autoclaving), screw caps with Teflon-coated sealing disks are useful (Fig. 3a). A special glass flask has been designed for sterilization and aseptic, anoxic storage of crude oil without loss of volatile components (Fig. 3b; [5]).

Hydrocarbons from stocks may be taken up with anoxic (N_2-gassed) syringes and added to the cultures, for instance, by injection through the stoppers (ideally as shown in Fig. 3c). The syringes should have plungers with plastic or Teflon sealing. Rubber-sealed plungers may be difficult to move after they have been in contact with liquid hydrocarbons.

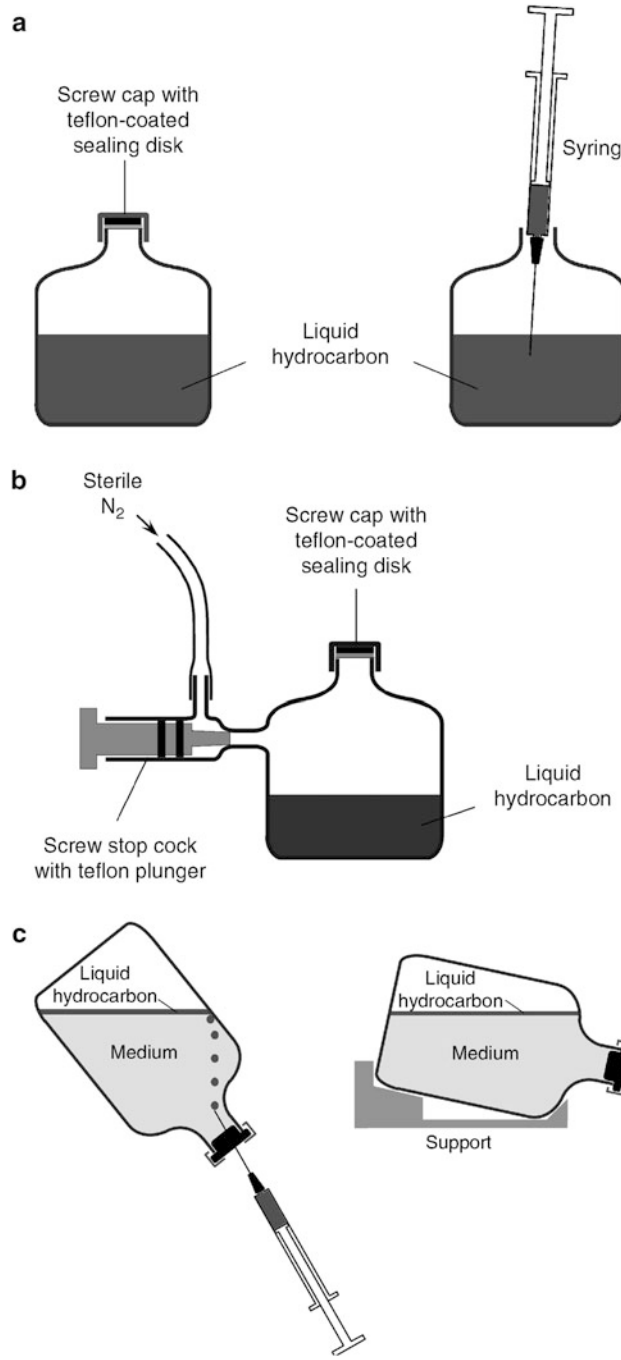

Fig. 3 Possibilities to handle liquid hydrocarbons as growth substrates. (**a**) Use of an autoclaved hydrocarbon solution. (**b**) Advanced method for anoxic maintenance of a sterile hydrocarbon. (**c**) Special method to add a liquid hydrocarbon to medium without coming into contact with the stopper

The problem of low solubility can be minimized by providing a large contact area between the medium and the overlying hydrocarbon phase. For this purpose, tubes or bottles (preferentially flat bottles) are incubated horizontally. This enlarges the surface area and minimizes diffusion distances between the hydrocarbon phase and the bacteria in the medium. Flat bottles (not shown) are particularly useful; however, they may not stand pressure. In a culture with hexadecane, α-cyclodextrin was used to improve growth [6]. Cyclodextrins possess hydrophobic interiors and form inclusion compounds with several hydrocarbons which in this way are "transported" into the aqueous phase.

The toxicity can in principle be minimized by adding extremely small amounts to keep the hydrocarbon concentration below saturation. However, such amounts are often below 1 mg l^{-1} and therefore yield only marginal cell growth. It is therefore much easier to provide such hydrocarbons from a dilute solution (often 1–20%, v/v) in an inert hydrophobic carrier. The overlying carrier phase then acts as a reservoir of the hydrocarbon substrate that is permanently provided at nontoxic concentration. Colorless refined mineral oil (paraffin oil, pharmaceutical grade; not useful for cultures that degrade long-chain alkanes), 2,2,4,4,6,8,8-heptamethylnonane, and pristane have been applied as carriers.

Adsorption of hydrocarbons to stopper material can be minimized or prevented in several ways. Stocks of sterile hydrocarbons can be kept in bottles with screw caps with Teflon-coated sealing disks. For culture tubes and bottles, Teflon-coated stoppers may be used; however, they are not easily available and they may be only fabricated by few companies upon special request. Even if needles penetrate these stoppers, the areas exposed to the hydrocarbon remain relatively small, and adsorption is much slower than at an unprotected stopper surface. In any case, stoppered culture tubes and bottles containing hydrocarbons should be kept in near-horizontal position so that the hydrocarbon phase is not in contact with the stopper. This is achieved by keeping the orifice always lower than the medium level (Fig. 3c). If the tube or bottle containing the hydrocarbon phase is initially in an upright position (which is usually the case), inversion to the horizontal position necessarily brings the hydrocarbon phase in contact with the stopper. Shaking the tube or bottle (causing a transient water-hydrocarbon emulsion) while it is simultaneously being inverted can avoid adherence of large hydrocarbon droplets to the stopper. An elegant approach is to add the hydrocarbon to the horizontal bottle through the stopper by means of an anoxic syringe; this can be done in such way that the hydrocarbon ascends to the medium surface without coming into contact with the stopper (Fig. 3c).

2.2.3 Solid Hydrocarbons

Solid hydrocarbons as pure substances have been used relatively rarely for cultivation of anaerobes. The best-known example is the aromatic hydrocarbon naphthalene [7] that may be added to a culture tube or bottle from an autoclaved (in closed bottle) stock as long as this is liquid. Certain inhibitory effects of naphthalene can be avoided, and better growth may be obtained after dissolution in a carrier phase (e.g., 20 mg ml^{-1}). Naphthalene has a noticeable vapor pressure and is slightly water soluble, so that supply of the slowly growing bacteria in the medium by diffusion is possible. Alkanes which are solid at room temperature (e.g., octadecane, $C_{18}H_{38}$; eicosane, $C_{20}H_{42}$; and higher) are insoluble and are essentially not available via diffusion into the medium. Such alkanes may be autoclaved and then added with pre-warmed pipettes to pre-warmed culture tubes or bottles. Tubes or bottles are rotated so as to distribute the alkanes and increase their surface area while they are solidifying.

2.3 Comments on Enrichment

For determination of the natural abundance of anaerobic hydrocarbon degraders, serial dilution of samples ("dilution to extinction") may be attempted, which represents a special enrichment strategy. However, apart from a few exceptions [8, 9], it is unknown how reliable this method is with the slowly growing anaerobic hydrocarbon degraders. So far, interest was mostly in principles of anaerobic hydrocarbon degradation (metabolic diversity and capabilities) rather than in natural abundances, and batch enrichments were commonly applied.

Media are provided with 5–10% (v/v) anoxic mud and the hydrocarbon. With the exception of the gaseous representatives, the consumption of hydrocarbons is difficult to quantify. Hydrocarbon-dependent microbial activity is detectable by monitoring the utilization of the electron acceptor in comparison to a hydrocarbon-free control. Sulfate reduction is easily detectable by formation of sulfide that can be detected in anaerobically withdrawn samples, for instance, with a copper-containing reagent [10]. Reduction of nitrate and of the intermediate nitrite can be measured by ion chromatography (with UV-detection). Often, a much simpler method, the measurement of formed gas (N_2) overpressure, is sufficient (Fig. 4).

2.4 Comments on Isolation

If consecutive subcultures have become sediment-free and show clear enrichment of cells (visible as turbidity and under the microscope), purification via serial agar dilution series [3] or liquid dilutions can be attempted. The agar and the liquid medium are overlaid with the hydrocarbon, as in the preceding enrichment. Both methods are promising if the hydrocarbon has a noticeable water solubility (e.g., gaseous alkanes, several aromatic hydrocarbons). With essentially insoluble hydrocarbons (e.g., hexadecane),

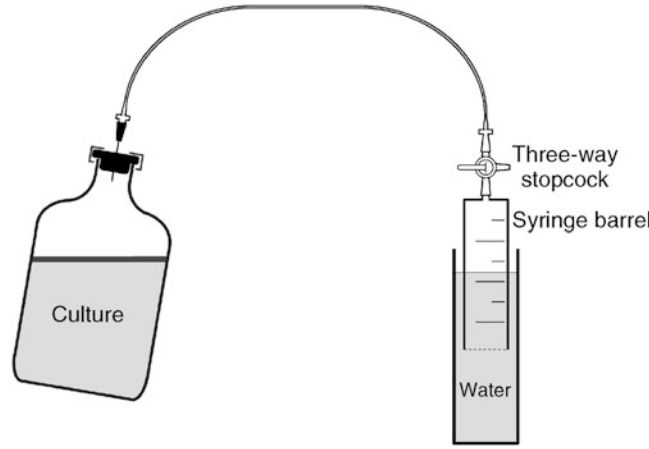

Fig. 4 Volumetric determination of formed N_2 as a simple measure of denitrification and nitrate consumption

cells that are not in direct contact with the hydrocarbon phase may not grow, and this is most likely the case for the few cells (or a single cell) at high dilution. Such failure of growth may be circumvented by adding alternative, soluble (polar) substrates. For instance, degraders of long-chain alkanes are expected to utilize also fatty acids (e.g., 1–2 mM sodium caproate, $C_5H_{11}COONa$). Pure cultures obtained with the alternative organic substrate are subsequently transferred to media with hydrocarbons to verify the ability for their utilization. Enrichment cultures with hydrocarbons often harbor accompanying bacteria that do not utilize the hydrocarbons (they presumably utilize excreted by-products) but usually grow faster with the non-hydrocarbon substrate added for isolation. Selection of hydrocarbon degraders with non-hydrocarbon compounds in liquid enrichment cultures is essentially impossible; they soon select for different degraders of non-hydrocarbons.

2.5 Disposal of Hydrocarbon Cultures

Old hydrocarbon cultures which are no longer in use belong to the more "problematic" types of microbiological waste. Due to the presence of an insoluble and possibly somewhat toxic hydrocarbon phase, old cultures should not be emptied into a regular sink. Especially cultures grown with crude oil or petroleum fractions would be problematic in regular wastewater. On the other, collection of the entire culture volumes for special disposal may soon result in big waste volumes. It is therefore recommended to collect old autoclaved hydrocarbon cultures in a separatory funnel. Toxic hydrogen sulfide from sulfate reduction should be oxidized by slow addition of H_2O_2 ("titration"). The aqueous phase can then be emptied into a regular sink (if there are no ingredients of high environmental concern), while the hydrocarbon phase is collected in a waste bottle for special disposal.

If cultures additionally contain mud (sediment), which usually harbors hydrocarbon droplets, the whole culture may be centrifuged. Because centrifuge beakers become "oily," a set of these should be kept separately from other beakers and be used only for hydrocarbon work. The supernatant (aqueous medium and hydrocarbon phase) is then decanted into the separatory funnel (see above). The mud pellet is removed by means of a spoon, allowed to dry in a bin under the fume hood, and disposed as special waste.

Stoppers contaminated with hydrocarbons may be used for subsequent cultures with the same hydrocarbon, or should be disposed.

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Enrichment and Isolation of Metal Respiring Hydrocarbon Oxidizers

Adam J. Williamson and John D. Coates

Abstract

The energy conservation strategies of plants and animals are exceedingly homogeneous in comparison with those of bacterial cells. Among the wide variety of mechanisms that exist to provide energy and electrons to support bacterial life, the use of insoluble metallic elements as electron acceptors is one of the most interesting. The prevalence of metallic elements in the minerals that comprise soils and sediments raises the possibility that metal respiring organisms play a significant role in a variety of biogeochemical transformations. The environmental abundance of metals also inspires the hope that cells capable of coupling metal respiration to the oxidation of contaminant hydrocarbons can be captured, studied, and manipulated to enhance biodegradation processes. Indirect mechanisms of iron cycling may also play a role in hydrocarbon degradation. Biogenic Fe(II) bearing minerals such as magnetite, as well as reactive Fe(II) sorption on crystalline iron oxides, have also been shown to dechlorinate carbon tetrachloride and reduce nitroaromatic compounds. This protocol provides an overview of anaerobic culturing and specific techniques for the enrichment and isolation of metal respiring hydrocarbon oxidizers from environments of interest.

Keywords: Anaerobic, Biogeochemistry, Culturing, Iron

1 Diversity of Hydrocarbon Respiring Metal Reducers

Anaerobic hydrocarbon oxidation is not only limited to Fe(III) rich environments but has also been shown to be coupled to the reduction of other metals and metalloids [1, 2]. Mn(IV) and As (V) reducing bacteria are diverse and ubiquitous in natural and anthropogenic sites and therefore should also be explored when seeking to isolate novel metal-reducing hydrocarbon-oxidizing microorganisms. Recent studies with hyperthermophilic Fe(III)-reducing hydrocarbon oxidizers may also be applied to in situ bioremediation of deep, high temperature contaminated sediments [3].

An increasing number of bacterial isolates are known to couple hydrocarbon oxidation to metal reduction. Six members of

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Table 1
Fe(III)-reducing hydrocarbon oxidizers

Characteristic	1	2	3	4	5	6	7	8	9	10
Morphology	Rod	Rod	Rod	Straight rod	Slight curved rod	Slight curved rod	Slight curved rod	Rod	Slight curved rod	Irregular cocci
Motility	✗	✗	✗	✗	NS	✓	✓	✓	✓	✓
Spore forming	✗	✗	✗	✗	✗	✗	NS	✓ endospores	✓ ellipsoid, terminal	NS
pH opt. (range tested)	0 (NS)	0 (NS)	6.5	6.6–7 (6.6–7.5)	NS	7.2 (NS)	7.3 (6.6–9)	7.2 (NS)	6.6–7 (6.6–7.5)	7
Temp opt. (range tested)	30–35 (NS)		35	25–32	30 (NS)	30 (16–40)	25–30 (21–40)	30 (NS)	30	85
Salinity opt. (range tested)	0 (NS)	0 (NS)	0 (0–1)	0 (NS)	NS	0 (NS)	0 (NS)	NS	0 (NS)	2
DNA G + C mol%	56.6	57.4	58.4	54.4	NS	60.9	NS	NS	47.7	44.1
Metal electron acceptor										
Fe(III)-oxide (ferrihydrite)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Fe(III)-citrate	✓	✓	✓	✓	✗	✓	✓	NS	✓	✓
Fe(III)-NTA	NS	NS	NS	NS	✓	NS	✓	NS	NS	NS
Fe(III)-pyrophosphate	NS	NS	NS	NS	✓	NS	✓	NS	NS	NS
Mn(IV)	✓	✓	NS	✗	NS	✓	✓	NS	✗	NS
As(V)	NS	NS	NS	NS	NS	NS	NS	✓	NS	NS
Aromatic hydrocarbon electron donor										
Benzene	✓[a]	NS	NS	NS	✓	NS	NS	NS	NS	✓
Ethylbenzene	✗	NS	NS	NS	NS	NS	✓	NS	NS	NS
Toluene	✓	✓	NS	✓	✓	✗	✓	✓	✓	NS
Phenol	✓	✓	✓	NS	NS	✓	✓	✓	✓	✓
Benzyl alcohol	✓	NS	NS	✓	NS	NS	NS	NS	✓	NS

Benzaldehyde	✓	✓	NS	✓	NS	✓	✓	NS	✓	NS
p-hydroxybenzaldehyde	✓	NS	NS	NS	NS	NS	NS	NS	NS	NS
Benzoate	✓	✓	NS	✓	✓	✓	✗	✓	✓	✓
o-hydroxybenzoate	✗	NS	NS	NS	NS	NS	NS	NS	NS	NS
m-hydroxybenzoate	✗	NS	NS	NS	NS	NS	NS	NS	NS	NS
p-hydroxybenzoate	✓	NS	NS	NS	NS	NS	✓	NS	NS	NS
o-crescol	NS	NS	NS	✗	NS	NS	✗	NS	✓	NS
m-crescol	✗	NS	NS	✓	NS	✓	✓	NS	✗	NS
p-crescol	✓	NS	NS	✓	NS	✗	✓	NS	✗	NS
o-xylene	✗	NS	NS	NS	NS	NS	✗	NS	✓	NS
m-xylene	✗	NS	NS	NS	NS	NS	✗	NS	NS	NS
p-xylene	✗	NS	NS	NS	NS	NS	✗	NS	NS	NS

(1) *G. metallireducens* [4], (2) *G. grbiciae* [5], (3) *G. hydrogenophilus*, (4) *G. toluenoxydans* [6], (5) *Geobacter* strain Ben [7], (6) *G. soli* [8], (7) *Georgfuchsia toluolica* [9], (8) *Desulfosporosinus* sp. Y5 [1], (9) *Desulfitobacterium aromaticivorans* [6], and (10) *Ferroglobus placidus* [3]

[a]Only coupled to Fe(III)-citrate

NS not stated in original manuscript

Geobacter have been isolated to date (Table 1): *G. metallireducens* [4], *G. grbiciae* [5], *G. hydrogenophilus* [5], *G. toluenoxydans* [6], *Geobacter* strain Ben [7], and *G. soli* [8]. A novel taxon of the Betaproteobacteria, *Georgfuchsia toluolica*, has also been identified to degrade several aromatic hydrocarbons coupled to a range of terminal electron acceptors including Fe(III)-NTA, Fe(III)-citrate, and amorphous Fe(III)-oxide.

Geobacter strain Ben is the only known bacterium capable of benzene oxidation coupled to the reduction of ferrihydrite [7]. In the same study, workers also demonstrated that *G. metallireducens* was capable of anaerobic benzene oxidation coupled to Fe(III)-citrate reduction. *Geobacter* species are ubiquitous in natural and engineered environments and stimulating Fe(III) reduction in contaminated environments may be a potential route for anaerobic benzene degradation. Furthermore, *G. metallireducens* is genetically tractable and genetic modifications of the hydrocarbon oxidation pathway may elucidate mechanisms of anaerobic hydrocarbon degradation and lead to novel isolation strategies.

Aromatic hydrocarbon degradation has also been observed outside the *Proteobacteria*. *Desulfosporosinus* sp. Y5 was isolated from an arsenic and hydrocarbon contaminated site and represents the first Gram-positive and only current microorganism capable of aromatic hydrocarbon oxidation coupled to As(V) reduction [1]. Stable isotope probing (SIP) has demonstrated that a novel clade of Gram-positive Fe(III)-reducing bacteria are prevalent in contaminated aquifers [10]. The enrichment culture derived from this work was capable of benzene oxidation and subsequent studies have demonstrated Fe(III) reduction coupled to monoaromatic and nonsubstituted polyaromatic hydrocarbon degradation by enrichment cultures [10–12]. However, isolation strategies have been largely unsuccessful, with only one isolate documented to date, *Desulfitobacterium aromaticivorans* [6]. *D. aromaticivorans* is the only known Gram-positive Fe(III)-reducing aromatic hydrocarbon-oxidizing bacterium and the first microorganism to couple Fe(III) reduction to *o*-xylene oxidation. Spore formation by these Gram-positive organisms may also give a significant physiological advantage in dynamic systems that are exposed to more hostile environmental conditions.

Two new genera of the Class Archaeglobi have recently been found to have a potential for metal reduction coupled to aromatic hydrocarbon reduction. For a comprehensive review of the Class Archaeglobi, see [13]. *Ferroglobus placidus* is currently the only known hyperthermophilic anaerobic euryarcheon capable of aromatic hydrocarbon oxidation coupled to Fe(III) reduction [3, 14]. *Geoglobus* represents another novel genus of the Class Archaeglobi with the potential for aromatic hydrocarbon degradation and two species have been isolated to date, *Geoglobus acetivorans* and *Geoglobus ahangari*. Both *G. acetivorans* and *G. ahangari* are

capable of oxidizing various simple and more complex organic substrates, coupled to their obligate dependence on Fe(III) reduction [15, 16]. Interestingly, genome analysis of *G. acetivorans* has indicated the presence of metabolic pathways for anaerobic oxidation of aromatic compounds and n-alkanes, although this has not yet been supported by laboratory studies [17]. The potential of *Geoglobus* species to oxidize aromatic hydrocarbons therefore clearly warrants further work as these species may play an important role in biogeochemical cycling in hydrothermal vents.

Accordingly, the prescriptions contained herein for the isolation and characterization of hydrocarbon-oxidizing metal respirers are best regarded as a starting point rather than a definitive guide. Innovative approaches that account for the principles underlying these recommendations, but also explore new methodological territory, are encouraged. To facilitate such exploration, a discussion of general principles precedes a more detailed explanation of specific technique. Because of the difficulty of enriching for both physiological abilities simultaneously, techniques for hydrocarbon oxidizers and metal reducers are presented separately, with more detail on the latter (for an excellent review of the former, the reader is referred to [18]). Descriptions of media and other useful techniques are included at the end of the chapter.

Two of the most important tasks in characterizing hydrocarbon-oxidizing metal respirers are linking changes in oxidant concentration to changes in reductant concentration and establishing that such changes are physiologically rather than chemically mediated. Constraints on experiments to determine these facts can include low hydrocarbon concentrations required to avoid toxicity; technical difficulty of accurately measuring small changes in metal concentrations; metal species contamination; and long reaction times.

2 Anaerobic Culturing

Nitrogen and carbon dioxide gases used for anaerobic culturing may be contaminated with small amounts of molecular oxygen. This problem can be minimized by passing the gases through heated copper filings (350°C) previously reduced by exposure to hydrogen gas ([19, 20]; Fig. 1). The culturing medium is heated to drive off residual oxygen and then sparged as it cools with the appropriate gases, whose relative partial pressure can be adjusted using flow meters. Flowing the same gas through the headspace during sparging and dispensing prevents the moving liquid from drawing air into the vessel during stirring. Including a chemical reductant in the medium will help scavenge any residual oxygen and protect against its re-introduction during dispensing, amendment, or inoculation [21].

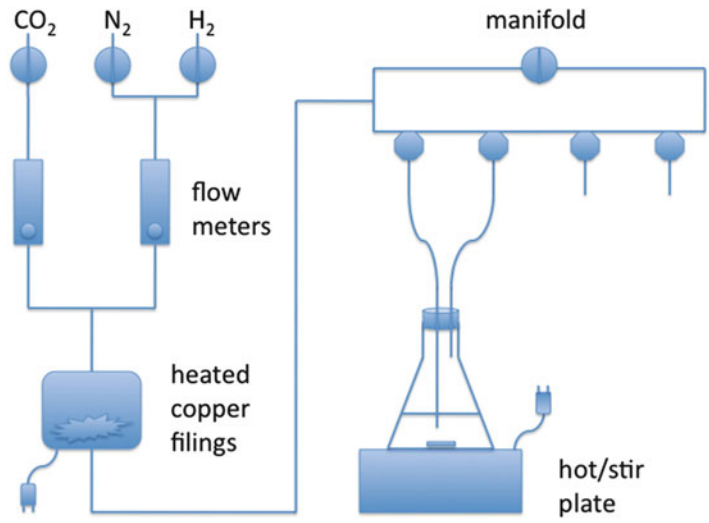

Fig. 1 Schematic diagram of equipment used for anaerobic media preparation

3 Hydrocarbon-Oxidizing Bacteria

For initial enrichments, liquid from the sampled environment can be de-aerated and sterilized for use as media. Otherwise, the media may be formulated to mimic the environment but also include an array of vitamins and trace minerals to support any unusual chemistry underlying anaerobic hydrocarbon transformation reactions.

When used as a sole source of carbon for the enrichment and isolation of anaerobic cells, hydrocarbons can pose unique challenges due to their toxicity, volatility, and insolubility (reviewed in [18]). Over the last two decades a great number of methodological innovations for the cultivation of anaerobic hydrocarbon-degrading bacteria were developed by Fritz Widdel's group at the Max Planck Institute, Bremen, Germany [22]. Hydrocarbons must be supplied in sufficient quantity to support cell growth, but below the threshold at which they inhibit growth. Methods to trap hydrocarbon molecules with an inert organic phases such as 2,2,4,4,6,8,8-heptamethylnonane or mineral oil have proven useful [23]. Amberlite XAD7 (0.3 g) is often used to strongly bind aromatic hydrocarbons to maintain a low concentration in media to avoid toxicity [6]. In such a case, the movement of hydrocarbons into the aqueous phase becomes dependent on removal by cells and is limited by solubility. De-aeration and sterilization of the hydrocarbon stock and carrier phase by autoclaving (less volatile hydrocarbons) or with solvent-resistant filters (0.2 μm pore size; more volatile hydrocarbons) are helpful in preventing contamination by molecular oxygen or cells not native to the environment being sampled, respectively. The volatility of many hydrocarbons has also been exploited by researchers who supply the substrate in the

vapor phase, allowing gas-liquid partitioning to deliver the hydrocarbons to cells [24].

When supplied in an inert overlay, the hydrocarbon of interest generally comprises 2% of the volume of the carrier phase, but its concentration can easily be monitored by gas chromatography and replenished if necessary [18]. To supply the hydrocarbon as a vapor, liquid hydrocarbon may be placed in an open container inside a larger airtight vessel containing cultures in Petri dishes [24]. The volume of liquid hydrocarbon should be set such that the final concentration in the culture medium does not exceed inhibitory levels. Different groups of bacteria have different tolerances, but Davidova and Suflita report that 150 μM benzene or 500 μM toluene generally do not impair the primary biochemical processes of interest (2005). Especially in the case of the more recalcitrant hydrocarbons, it may be useful to use water-soluble hydrocarbon derivatives such as 4-chlorobenzoate (0.5 mM) as an electron donor to allow faster growth [25].

4 Metal-Reducing Bacteria

Oxidized metallic elements such as iron that can serve as terminal electron acceptors often exist in insoluble forms, especially at pH values that support diverse bacterial communities. As a result, it can be helpful to include a chelating molecule such as nitrilotriacetic acid (NTA), ethylenediaminetetracetic acid (EDTA), citrate, or naturally occurring organic matter such as humic acids to distribute the metal throughout the liquid phase at circumneutral pH [26–28]. Alternatively, the addition of an electron shuttling agent such as 2,6-anthraquinone disulfonate (AQDS) at low concentration (100 μM) has been shown to significantly improve hydrocarbon degradation. Humic acids also contain redox active quinone moieties that have been linked with enhanced hydrocarbon oxidation [29]. However, one must bear in mind that the chelator or electron shuttling agent itself may be toxic or act as an alternative and more labile electron donor for the microbial enrichment. This is often the case with ligands such as citrate. The molecular context of the metallic element should also be considered. The structure and composition of the mineral species can affect the geometry of electrostatic microenvironment and the redox potential of the metal, potentially determining the types of cells that can access it [30].

5 Direct Isolation

Dissimilatory Fe(III)-reducing bacteria can be directly isolated from a broad diversity of environments using a modified shake tube method. The media of choice uses Fe(III) chelated with nitrilotriacetic acid (10 mM) as the sole electron acceptor and either

the hydrocarbon of choice or any other non-fermentable electron donor such as H_2 (101 kPa), acetate (10 mM), or lactate (10 mM). If H_2 is used a small amount of a suitable carbon source, such as 0.1 g/L yeast extract, should be added to the medium. The media should be further amended with $FeCl_2$ (2.5 mM) as a reductant. Freshly collected samples are serially diluted to 10^{-9} in this medium. Aliquots (7 mL) of the respective dilutions are transferred anaerobically into anaerobic pressure tube containing 3 mL of sterile molten noble agar (Difco) (4% wt./vol.) at 55°C under a gas phase of N_2-CO_2 (80–20, vol./vol.). The sample is mixed by inverting several times and then solidified by plunging into an ice bath. The solidified dilutions are incubated inverted. Colonies of Fe (III)-reducing bacteria should be visible in the lower dilutions (10^{-1}–10^{-3}) after 2 weeks incubation. These can easily be recognized as small (0.5–1 mm diam.) pink colonies surrounded by a colorless clear zone in the orange-colored agar. In an anaerobic glove bag colonies can be picked as plugs using a sterile Pasteur pipette, and transferred into fresh anaerobic medium (5 mL) with Fe(III)-NTA (10 mM) and a suitable electron donor. These nascent cultures should be further amended with $FeCl_2$ (2.5 mM) as a reductant. Active cultures can easily be recognized after 2–4 weeks incubation by the color change in the medium from orange to colorless with a white precipitant in the bottom of the tube. Active cultures should be transferred through a second dilution shake tube series to ensure isolation.

6 Selective Enrichment and Isolation

Dissimilatory Fe(III)-reducing bacteria can be selectively enriched from diverse habitats in basal media using various forms of Fe(III) as the sole electron acceptor with either H_2 or simple organic acids as the electron donor. Insoluble amorphous Fe(III)-oxide is the preferential form for enrichment initiation as there are no organic complexing agents which could potentially be biodegradable and serve as a competing carbon and energy source for fermentative bacteria. While the carbon source supplied in Fe(III) enrichments is the most important variable that can shift a microbial community, the type of Fe(III)-oxide and sediment dilution can also influence community composition. For instance, acetate will enrich for *Geobacter* type species that predominantly grow on ferrihydrite. However, the addition of glucose or lactate may lead to a more versatile Fe(III)-reducing community that can reduce a range of Fe(III)-oxides, as well as promoting fermentative and sulfate reducing bacteria [31]. Samples collected from the field should completely fill any vessel in which they are collected to exclude air in the headspace. These should be sealed, transported back to the laboratory, and used immediately. If not used immediately, samples

should not be frozen but may be stored at 4°C for short periods. Enrichments should be initiated with inoculum sizes of 10% by weight of the culture volume. Incubations should be carried out at environmental temperatures depending on the source of the sample.

6.1 Enrichment for Geobacter *Species*

Geobacter species are mesophilic, complete-oxidizing, obligate anaerobes. Amorphous Fe(III)-oxide (30 mM) is a suitable electron acceptor for *Geobacter* species with acetate (2 mM) as the sole electron donor. Enrichments are generally incubated at temperatures of 15–30°C. Positive enrichments can be visually identified by a color change in the amorphous Fe(III) from orange-brown to black as the iron is reduced. If left for an extended period after complete reduction of the Fe(III) has taken place (1–2 weeks), the crystalline iron mineral magnetite (Fe_2O_3) will form which can readily be identified by its magnetic properties (Fig. 2). Initial enrichments usually take 1–2 weeks at 30°C. Initial positive enrichments should be transferred as soon as the iron precipitant in the media has turned black. Inoculum transfers into fresh media should be 10% of the culture volume. Sequential transfers should be done three times to remove residual particulates and biodegradable organics associated with the original sample. At this stage, the enrichment may be transferred into basal media amended with a soluble form of Fe(III) such as Fe(III)-NTA (10 mM). Growth can be recognized by a color change in the media from transparent orange to colorless and the formation of $FeCO_3$, which is seen as a white precipitant at the bottom of the culture vessel. Cell yields are generally poor, and an optical increase in cell density will not be apparent. Once growth has been achieved on Fe(III)-NTA, the

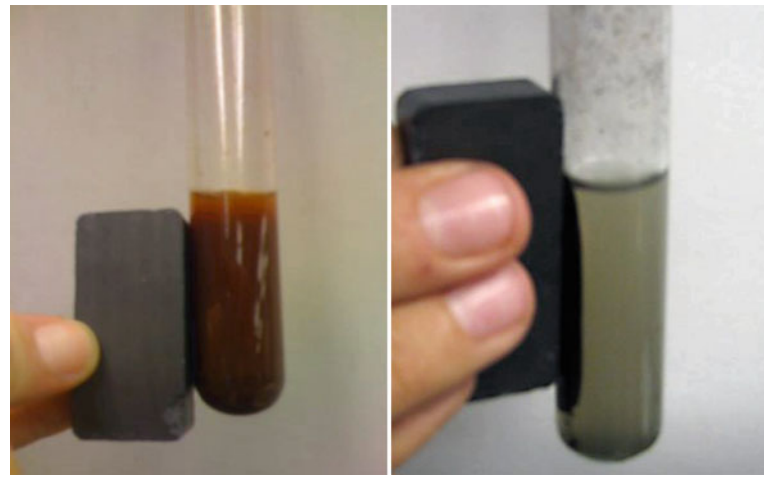

Fig. 2 Iron in the culturing medium that has been converted into magnetite will be attracted by a magnet through the wall of the culturing tube (Photo credit: J. Cameron Thrash. Hand model: Kelly Wrighton)

enrichment should be transferred to solid media with acetate (2 mM) and Fe(III)-NTA (10 mM) as the sole electron donor and acceptor, respectively, amended with 2% by weight noble agar (Difco). Colonies of Fe(III)-reducing bacteria can readily be recognized on the surface of agar plates as small white colonies surrounded by a colorless halo in tan-brown colored agar. Colonies should be picked with sterile glass Pasteur pipettes in an anaerobic glove box and restreaked onto fresh solid media. After incubation several of the restreaked colonies should be picked and used to inoculate a single tube of fresh anaerobic Fe(III)-NTA media. It is a good idea to amend these nascent cultures with $FeCl_2$ (2.5 mM) as a reducing agent. A viable Fe(III)-reducing culture should be obtained after 7–10 days.

6.2 Enrichment for Desulfuromonas *Species*

Desulfuromonas species can readily be isolated from marine environments with Fe(III)-oxide as the sole electron acceptor. The APW medium (see media recipes) is a suitable enrichment and growth medium for marine samples. Samples should be freshly collected and transported back to the laboratory in sealed vials that are filled to capacity to exclude any O_2. Acetate (10 mM) is a suitable electron donor and amorphous Fe(III)-oxide is the electron acceptor of choice. Enrichments should be initiated as outlined above for *Geobacter* species. Active Fe(III)-reducing enrichments should be observed within 7–14 days at 30°C. Active cultures should be passed through at least four transfers of this medium prior to transfer into medium with a soluble Fe(III) source. Fe(III) chelated with citrate is the soluble iron form of choice as Fe(III)-NTA precipitates the bound Fe(III) at high salinities. The highly enriched culture can then be transferred into APW medium with acetate and soluble Fe(III)-citrate (50 mM) as the sole electron donor and acceptor, respectively. Growth and Fe(III) reduction can be visually recognized as the medium turns from a deep red transparent color to dark green and finally to a light green with a white precipitate of $FeCO_3$ visible at the bottom of the tube. The enrichment can then be transferred onto solid medium with Fe(III)-citrate as the electron acceptor. Fe(III)-reducing colonies will be visible after 7–14 days and can be easily recognized as small pink colonies surrounded by a green halo in the dark red medium. Colonies can be picked as plugs using a sterile Pasteur pipette and restreaked onto fresh agar plates. Several colonies can be isolated from these latter plates and transferred into fresh liquid medium. Active liquid cultures of the isolated Fe(III)-reducers should be apparent after 5–10 days.

6.3 Enrichment for Shewanella *Species*

Shewanella species have been isolated from both freshwater and estuarine environments. Amorphous Fe(III)-oxide (30 mM) is a suitable iron form for enrichments of *Shewanella* species. Lactate (20 mM) or H_2 (101 kPa) are the electron donors of choice. If H_2 is used, the medium should be amended with yeast extract (0.1 g/L) as a suitable carbon source. Positive enrichments can be identified as

outlined above for *Geobacter* and *Desulfuromonas* species. Once a robust enrichment has been obtained by four or five passages through an enrichment series the active culture can be streaked on tryptic soy broth (TSB) (Difco) agar plates and incubated aerobically at 30°C. Colonies of *Shewanella* species will appear as pink smooth colonies, 2–4 mm in diameter, after 2–3 days of incubation. Colonies can be picked and restreaked on TSB plates to further purify the cultures. All isolates obtained in this fashion should be transferred back into anaerobic medium with Fe(III)-oxide and lactate or H_2 as the electron acceptor and donor, respectively.

6.4 Enrichment for Geothrix *Species*

Recent studies [32, 33] have shown that *Geothrix* species may be one of the dominant Fe(III)-reducers found in mesophilic freshwater environments. These species are relatively slow growing strict anaerobes. Fe(III)-pyrophosphate (10 mM; Pfaltz and Bauer, Inc.) is the electron acceptor of choice for their isolation. Enrichment for *Geothrix* species is similar to that for *Geobacter* with acetate (10 mM) and amorphous Fe(III)-oxide as the sole electron donor and acceptor, respectively. Incubation periods should be lengthened to 3–4 weeks for each stage of the enrichment to allow the slow growing *Geothrix* species to compete. After five passages of the enrichment through an enrichment series the active culture can be streaked onto anaerobic plates of Fe(III)-pyrophosphate (10 mM) medium with acetate (10 mM) as the sole electron donor and amended with 2% (wt./vol.) noble agar (Difco). The plates should be incubated anaerobically for 3–4 weeks at 30°C. Fe(III)-reducing colonies can easily be recognized as small (1–2 mm diam.) white colonies surrounded by a colorless halo in light green agar. The colony is encrusted with a white mineral precipitate, which is presumably vivianite (Fe_3PO_4). Colonies should be picked and restreaked onto fresh agar plates to obtain isolates. After incubation, several of the restreaked colonies should be picked and used to inoculate a single tube of fresh anaerobic Fe(III)-pyrophosphate media. The liquid medium should be amended with $FeCl_2$ (2.5 mM) as a reducing agent. Active Fe(III)-reducing cultures should be apparent after 3–4 weeks and can be easily recognized by a color change in the medium from light green to colorless and the appearance of a white precipitate at the bottom of the tube.

6.5 Enrichment for Georgfuchsia *Species*

Recent work using SIP has demonstrated that these organisms may be more diverse than first thought [12]. However, little is known about these species and only one isolate has been recovered to date [9]. *Georgfuchsia* is optimally and routinely grown with toluene (0.25 or 0.5 mM) and nitrate (10 mM), with 330 μM toluene oxidized in 6 days. Good growth is also observed with Fe-NTA(10 mM) (7 days for 215 μM toluene) and MnO_2 (20 mM), but slower growth occurs with amorphous Fe(III)-oxide (40 mM). Enrichments are conducted in pH 7.3 phosphate-buffered bicarbonate medium (see media recipes) (40 mL) with cysteine (1 mM) as a reducing agent at 30°C.

MnO_2 reduction is accompanied by a color change of brown to white precipitates. Subsequent 10% transfers should be carried out into fresh media. For isolation, enrichments should be streaked onto solid media (1.2% agar) with toluene and nitrate and incubated at 30°C. Colonies are red-brown in color, round, lens shaped with a 0.5–1 mm diameter. Cells are motile, Gram-negative straight to slightly curved rods, 0.5–0.6 μm wide, and 0.8–1.2 μm in length, with a tendency to form chains. Growth was observed at pH 6.6–9 (optimum 7.3) and temperature of 20–37°C (25–30°C).

6.6 *Enrichment for* Geoglobus *and* Ferroglobus *Species*

The Class Archeoglobi is comprised of three genera: *Archaeglobus*, *Geoglobus*, and *Ferroglobus*; all of which are obligate anaerobic thermophiles or hyperthermophiles and have all been isolated from hydrothermal environments from various depths, posing a challenging sampling strategy, enrichment, and isolation. Furthermore, the traditional plating method is not as effective at high temperatures therefore optical tweezers are typically used to pick individual colonies from enrichment cultures. Currently, only *Ferroglobus* and *Geoglobus* species are known to be capable of Fe(III) reduction and *Ferroglobus placidus* is currently the only member of the Archaeglobi family that is also capable of hydrocarbon oxidation. *F. placidus* was enriched from shallow (1 m depth) marine hydrothermal system at Vulcano, Italy by incubating 1 mL aliquots of the sand/water mixture from the site and incubated in *Ferroglobus* media (see media recipes) at 85°C with shaking and FeS and NO_3^- as electron donor and acceptor, respectively. Subsequent isolation was performed using optical tweezers from the enrichment culture and growth was observed at pH 6–8.5 (optimum 7), 65–95°C (85°C), and 0.5–4.5% (2%) salinity. *F. placidus* has now been shown to grow with acetate (10 mM), phenol (0.5 mM), benzoate (1 mM), or benzene (2 mM) as the electron donor and Fe(III)-citrate (56 mM) or ferrihydrite (100 mM) as the electron acceptor. To adapt *F. placidus* to grow on benzene, cultures should be initially grown with added hydrogen (pH_2 101 kPa), and then subsequently transferred into medium with no hydrogen.

Geoglobus acetivorans was isolated from a hydrothermal sample collected on the Mid Atlantic ridge [17]. Active hydrothermal chimney fragments were stored with in situ seawater and stored at 4°C. The enrichment was initiated with a 10% wt./vol. transfer into anaerobic bicarbonate buffered medium at 65°C (see media recipes), with acetate (18 mM) as electron donor and amorphous Fe(III)-oxide as electron acceptor. *G. acetivorans* initially did not grow on Fe(III)-citrate but adapted to grow slowly on this after 100 days of incubation. Efforts to isolate this microorganism using roll tubes with both Gelrite gellan gum and 2% agar at 65 and 82°C have been unsuccessful [16]. Instead, enrichments should be subsequently transferred several times under lithotrophic conditions at pH 6.5–6.8 and 80°C with H_2 and Fe(III) as electron donor/acceptor, respectively, until a single isolate remains.

7 Culture Maintenance

All mesophilic Fe(III)-reducing cultures can be maintained as frozen stocks at −70°C. The most reliable technique is to grow the culture in media amended with a soluble electron donor and acceptor such as acetate (10 mM) and fumarate (50 mM). Once a dense culture has been obtained, aliquots (1 mL) should be anaerobically transferred into small serum vials (10 mL) which have previously been gassed out with N_2-CO_2 (80–20; vol./vol.) and heat sterilized. The vials should be amended with an anaerobic glycerol solution (100 mL) (25% vol./vol.) mixed and frozen at −70°C. Frozen stocks should be checked regularly to ensure viability. There is limited information on the storage of the thermophilic *Geoglobus* and *Ferroglobus* species. *F. placidus* must be transferred every 2 days or stored in 5% DMSO on liquid N_2 at −140°C.

8 Stable Isotope Probing

SIP is increasingly being used to examine active anaerobic toluene degrading species in mixed cultures. ^{13}C, ^{15}N, or ^{18}O labeled hydrocarbons and their derivatives are added to experiments and are assimilated by the associated hydrocarbon-degrading microorganism. SIP directly targets DNA and thus identifies the bacteria involved in hydrocarbon degradation without the need to culture the bacteria involved. This in turn may assist in formulating isolation strategies. For a comprehensive review of DNA and RNA based SIP of hydrocarbon-degrading microorganisms see [34, 35].

9 Media Recipes

9.1 Freshwater Fe (III)-Oxide Basal Medium

Component	Amount
H_2O	1.0 L
Amorphous Fe(III)-oxide	30 mL
NH_4Cl	0.25 g
NaH_2PO_4	0.60 g
$NaHCO_3$	2.5 g (primary buffer with CO_2 below)
KCl	0.1 g
Vitamin solution	10 mL
Mineral solution	10 mL

- Split medium into tubes before sparging with 80% N_2 and 20% CO_2 (at least 6 min, the last minute with the stopper in place).
- Final pH should be 6.8–7.0
- Autoclave for 20 min @ 121°C
- After autoclaving add an appropriate electron donor from sterile anoxic stock.
- The amorphous Fe(III)-oxide can be replaced with alternative Fe(III) forms as needed.

9.1.1 Vitamin Solution

Component	Amount (mg/L)
Biotin	2
Folic acid	2
Pyridoxine HCl	10
Riboflavin	5
Thiamin	5
Nicotinic acid	5
Pantothenic acid	5
Vitamin B-12	0.1
p-aminobenzoic acid	5
Thioctic acid	5

9.1.2 Mineral Solution

Component	Amount (g/L)
Nitrotriacetic acetic acid (NTA)	1.5
$MgSO_4$	3.0
$MnSO_4 \cdot H_2O$	0.5
NaCl	1.0
$FeSO_4 \cdot 7H_2O$	0.1
$CaCl_2 \cdot 2H_2O$	0.1
$CoCl_2 \cdot 6H_2O$	0.1
ZnCl	0.13
$CuSO_4$	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	0.01
H_3BO_2	0.01
Na_2MoO_4	0.025
$NiCl_2 \cdot 6H_2O$	0.024
$Na_2WO_4 \cdot 2H_2O$	0.025

9.2 Freshwater Fe (III)-Citrate Medium

The above outlined basal medium can be prepared with 50 mM soluble Fe(III) chelated with citrate in replacement of the amorphous Fe(III)-oxide. The Fe(III)-citrate can be added from a sterile anaerobic stock (1 M) (see Fe(III)-citrate stock solution below) or directly into the basal medium as follows:

Component	Amount	Instructions
H_2O	1 L	
NaOH	3.4 g/L	Dissolve
Fe(III)-citrate	14 g/L	Boil and cool to room temperature to dissolve

- Check pH (adjust to 6.0)
- Add medium components outlined for basal medium above

9.3 Freshwater Fe (III)-Pyrophosphate Medium

The amorphous Fe(III)-oxide in the freshwater basal medium can alternatively be replaced with 3.0 g/L soluble Fe(III)-pyrophosphate (Pfaltz and Bauer) added directly to the basal medium as it is prepared. The resulting Fe(III) concentration will be 10 mM.

9.4 Freshwater Fe (III)-NTA Medium

Fe(III) chelated with nitrilotriacetic acid is only suitable for freshwater media as the Fe(III) will precipitate out of solution at elevated salinities. In addition Fe(III)-NTA should be filter sterilized and added from an anaerobic sterile stock (1 M) into heat sterilized media just prior to inoculation. Fe(III)-NTA stocks are prepared as outlined below.

9.5 APW Medium for Marine Isolates

Component	Amount
H_2O	1.0 L
Ferric citrate	13.7 g (dissolve by heat, cool, and adjust pH to 6.0 with NaOH)
$NaHCO_3$	2.5 g (primary buffer with CO_2 below)
Salt solution A	20 mL
Vitamin solution	10 mL
Mineral solution	10 mL

- Split medium into tubes before sparging with 80% N_2 and 20% CO_2 (at least 6 min, the last minute with the stopper in place).
- Autoclave for 20 min @ 121°C
- After autoclaving add 50 mL Salt Solution B and an appropriate electron donor from sterile anoxic stock
- Final pH should be 7.5–7.7
- The vitamin and mineral solution are as outlined above for freshwater basal medium.

9.5.1 Salt Solution A

Component	Amount (g/100 mL)
NaCl	24.0
NH_4Cl	5.0
KCl	1.17
KH_2PO_4	0.5
$MgSO_4 \cdot 7H_2O$	1.0
$CaCl_2 \cdot 2H_2O$	0.1

9.5.2 Salt Solution B

Component	Amount (g/100 mL)
$MgCl_2 \cdot 6H_2O$	21.2
$CaCl_2 \cdot 2H_2O$	3.04

10 Phosphate Buffered Bicarbonate Media for *Georgfuchsia* Species

Component	Amount
H_2O	1 L
K_2HPO_4	0.65 g
$NaH_2PO_4 \cdot 2H_2O$	0.2 g
NH_4HCO_3	0.44 g
$CaCl_2 \cdot 2H_2O$	0.11 g
$MgCl_2 \cdot 6H_2O$	0.10 g
$NaHCO_3$	3.73 g
4% wt./vol. fermented yeast extract solution	10 mL
Mineral solution	1 mL
Vitamin solution	1 mL

Fermented yeast extract is made by inoculating 5 g granular sludge from a sugar refinery in 2.1 g $NaHCO_3$ in 500 mL dH_2O with 20 g yeast extract at 37°C for 3–4 weeks, centrifuging, and filter sterilizing the supernatant.

11 Ferroglobus (FM) Media

Component	Amount
H_2O	1 L
KCl	0.34 g
$MgCl_2 \cdot 6H_2O$	4.3 g
NH_4Cl	0.24 g
$CaCl_2 \cdot 2H_2O$	0.14 g
NaCl	18 g
$NaHCO_3$	2.5 g
$K_2HPO_4 \cdot 3H_2O$	0.14 g
KH_2PO_4	0.5 g
Mineral solution	10 mL

Media should be prepared with N_2-CO_2 (80–20, vol./vol.). After autoclaving, add selected electron donor and acceptor, followed by $FeCl_2$ (1.3 mM), Na_2SeO_4 (30 μg/L), Na_2WO_4 (40 μg/L), APM salts (1 g/L $MgCl_2$, 0.23 g/L), and DL vitamins (10 mL/L) [36].

12 Bicarbonate Buffered Media for *Geoglobus* Species

Component	Amount
H_2O	1 L
NaCl	18 g
$MgCl_2$	4 g
KH_2PO_4	0.33 g
NH_4Cl	0.33 g
KCl	0.33 g
$MgCl_2 \cdot 2H_2O$	0.33 g
$CaCl_2 \cdot 2H_2O$	0.33 g
$NaHCO_3$	2 g
Peptone	10 g
Yeast extract	0.2 g
Mineral solution	1 mL

Prepare media under a CO_2 gas phase, adjust pH to 6.5–6.8 using 10% wt./vol. NaOH and autoclave for 1 h at 135°C. After

autoclaving, sterile acetate (18 mM) and amorphous Fe(III)-oxide (90 mmol/L) can be added.

13 Other Techniques for Working with Metal Reducers

13.1 Various Fe(III) Forms

13.1.1 Amorphous Fe (III)-Oxide Stock

- Dissolve 109 g/L $FeCl_3$
- Bring pH carefully to pH 7.0 with 10 M NaOH
- Centrifuge for 20 min at 3,000 × *g*
- Pour off supernatant
- Resuspend pellet in distilled water (500 mL) and repeat centrifugation
- Continue this washing procedure until Cl^- ion concentration is less than 1.0 mM
- Resuspend final pellet in 100 mL distilled water and store at 4°C
- Use 1 mL of stock solution in 9 mL basal medium

13.1.2 Fe(III)-NTA Stock (1 M)

Component	Amount (g/100 mL)
$NaHCO_3$	16.4
Nitrilotriacetic acid (sodium salt)	25.6
$FeCl_3$	27.0

- pH to 6.5
- Degas by sparging with N_2-CO_2 (80–20, vol./vol.) for 10 min
- Filter sterilize

13.1.3 Ferric Citrate Stock

Component	Amount (g/100 mL)
NaOH	6.8
Fe(III)-Citrate	28.0

- Heat to dissolve and cool.
- Degas with N_2 for 20 min and seal with a thick butyl rubber stopper
- Autoclave at 121°C for 15 min

13.2 Assaying Fe (III)/Fe(II)

13.2.1 Solutions

HCl	0.5 N
Ferrozine in 50 mM Hepes buffer, pH 7.0	1 g/L
Hydroxylamine	6.25 N

Standards should be prepared from $FeSO_4(NH_4)_2SO_4{\cdot}6H_2O$ (0–20 mM)

13.2.2 Reduced Iron Assay

- Add 0.1 mL sample (10–50 mM Fe) to 5 mL 0.5 N HCl
- After dissolution add 0.1 mL of the above to 5 mL ferrozine solution.
- Filter through a 0.2 mm filter and read absorbance at 562 nm.

13.2.3 Total Iron Assay

- Add 0.1 mL sample (10–50 mM Fe) to 5 mL 0.5 N HCl and allow it to dissolve.
- Add 200 mL of the hydroxylamine solution to the dissolved sample.
- After 1 h incubation at room temperature in the dark take 0.1 mL of above and add to 5 mL ferrozine.
- Read absorbance at 562 nm.

13.3 MnO_2 Synthesis

Mix equal amounts of 0.4 M $KMnO_4$ and 0.4 M $MnCl_2$ and adjust to pH 10 with NaOH. Wash the metal oxide suspension three times with dH_2O and suspend in dH_2O

13.4 Mn Colorimetric Assay [37]

13.4.1 Solutions

- 0.6 M ascorbic acid
- HACH alkaline cyanide reagent
- HACH PAN indicator solution
- Add 750 μL dH_2O and 50 μL to a 1.5 mL cuvette
- Add 250 μL sample and mix
- In the fume hood add two drops of alkaline cyanide reagent followed by 70 μL of PAN indicator solution
- Cover the cuvette with parafilm and mix
- Quantify on UV/Vis at 562 nm (Target conc is 0–5 ppm)

13.5 Most Probable Number Counts (MPNs)

MPNs for Fe(III)-reducing populations in freshwater environments can be performed with a slight modification to the media outlined above. Amorphous Fe(III)-oxide is the iron form of choice as no false positives result from the presence of easily biodegraded organics such as citrate present in the Fe(III)-citrate medium. A non-fermentable electron donor such as H_2, (101 kPa), acetate (2 mM), or lactate (5 mM) should be used. If H_2 is being used, yeast extract (0.1 g/L) should be added as a carbon source.

Basal freshwater medium as outlined previously

Amendment	Amount (per 1,000 mL)
Nitrilotriacetic acid (disodium salt) (4 mM)	0.94 g
Amorphous Fe(III)-oxide	30 mL

Dispense in 9 mL aliquots into pressure tubes and degas and then gas individually with N_2-CO_2 (80–20, vol./vol.) as

previously outlined. Autoclave at 121°C for 15 min. Just prior to use add $FeCl_2$ (0.1 mL) from a sterile anoxic stock (250 mM) as a reductant and 0.1 mL sodium pyrophosphate from a sterile anaerobic 10% (wt./vol.) aqueous stock solution to the initial dilution tubes. This will serve to release any cells adsorbed onto the soil/sediment particles and significantly improve the counts obtained. Tubes should be incubated at temperatures suitable to the original sample environment. Positive MPN tubes should be read after 60 days incubation and can be identified by measuring the Fe(II) content using the ferrozine assay. An initial visual screening of the tubes can be done by an observable color change in the iron precipitate from rust-brown to a dark black color.

13.6 Hydrocarbon Determination

Aromatic hydrocarbon concentrations can be determined with GC/MS. The concentration of aromatic hydrocarbons can be determined at the specific incubation temperature using Henry's law. Benzoate and phenol can also be determined using HPLC with a MeOH-H_2O (60:40%) and 0.1% H_3PO_4 eluent at an absorbance of 280 nm.

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Enrichment and Isolation of Chloroxyanion-Respiring Hydrocarbon Oxidizers

Tyler P. Barnum and John D. Coates

Abstract

Converging lines of research point to chloroxyanion-respiring hydrocarbon oxidizers as an important group of microorganisms that merit expanded isolation and characterization. Respiration of the chloroxyanions perchlorate (ClO_4^-) and chlorate (ClO_3^-), collectively termed (per)chlorate, is the only non-phototrophic process shown to produce molecular oxygen (O_2). Accordingly, strains that respire (per)chlorate while oxidizing hydrocarbons have been of longstanding interest for bioremediation of hydrocarbons in anaerobic environments (Coates et al., Nature 396(6713):730, 1998). Perchlorate also shows promise as a corrective to corrosive sulfidogenesis in oil reservoirs. The perchlorate ion is a putative competitive inhibitor of ATP sulfurylase, a key enzyme in the sulfate reduction pathway (Carlson et al., ISME J 9:1295–1305, 2014); (per)chlorate reduction can be coupled to the oxidation of sulfide into sulfur and sulfate (Gregoire et al., Environ Microbiol Rep 6(6):558–564, 2014); and in reservoirs amended with perchlorate, sulfate-respiring microorganisms could be competitively excluded by perchlorate-respiring hydrocarbon oxidizers (Engelbrektson et al., Frontiers Microbiol 5, 2014; Liebensteiner et al., Frontiers Microbiol 5(428), 2014). The discovery of mesophilic and hyperthermophilic (per)chlorate-reducing microorganisms that can consume hydrocarbons (Carlström et al., Appl Environ Microbiol 81(8):2717–2726, 2015; Coates et al., Nature 411(6841):1039–1043, 2001; Liebensteiner et al., Science 340(6128):85–87, 2013) emphasizes the potential for bioremediation and oil reservoir souring control and provides exciting motivation to characterize additional (per)chlorate-reducing microorganisms from similar environments.

This protocol provides an overview of anaerobic culturing and handling hydrocarbons (reviewed extensively in Davidova and Sulfita, Methods Enzymol 397(05):17–34, 2005) and specific techniques for the enrichment and isolation of chloroxyanion-respiring hydrocarbon oxidizers from several environments of interest.

Keywords: Azospira, Chlorate, Chlorite, Chlorite dismutase, Dechloromonas, Perchlorate

1 Anaerobic Culturing

In order to grow oxygen-sensitive strains and control oxygen availability in physiological studies, oxygen must be removed from culture media and prevented from reentering. Anaerobicity is achieved by replacing oxygen with inert gas in the media and headspace of sealed containers. The inert gas most commonly

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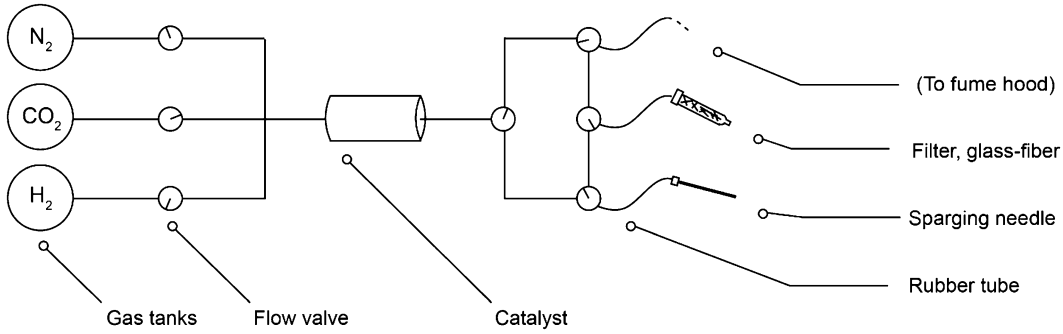

Fig. 1 A simplified schematic of an anaerobic gas station

used is nitrogen, but a mix of gases (e.g., 80% nitrogen and 20% carbon dioxide) may be more appropriate for certain growth conditions in which CO_2-bicarbonate is used as a pH buffer. Gas tanks contain trace amounts of oxygen that can be removed by a catalyst; heated copper filings previously reduced with hydrogen gas are a common configuration of many laboratory gas stations (Fig. 1). Oxygen is soluble in water at ambient temperature and pressure and can be replaced by boiling the solution, which lowers gas solubility, then cooling the solution to room temperature while sparging with oxygen-free gas. The solution should then be distributed into tubes or bottles under an oxygen-free gas phase and sealed. Balch tubes and serum bottles provide effective sealing when capped with butyl rubber stoppers and crimped. Upon sealing tubes and bottles, the gas can be exchanged using the laboratory gas station in order to remove any residual oxygen in the headspace or to replace the headspace with alternative gases, e.g., H_2 at specific concentrations.

Any media or solution added to anaerobic cultures should be sterilized by autoclaving or by passing through a 0.22-μm filter. Volatile or heat-sensitive compounds and small amounts of solution should be made anaerobic at room temperature by extensively flushing with nitrogen gas in a chemical fume hood (or repeatedly vacuuming and flushing) then filter-sterilized. Adding a chemical reductant such as sodium sulfide at low concentration (~100–500 μM) to scavenge trace oxygen is suggested for the enrichment and isolation of (per)chlorate-reducing microorganisms but not for physiological characterization. Higher concentrations (above 1 mM) of H_2S are known to be inhibitory to growth and should be avoided [1]. Whenever syringes are used to transfer media, the syringe should be flushed with glass fiber-filtered oxygen-free gas to remove oxygen from the interior. Using these approaches, physical manipulation of samples and cultures can be performed on the benchtop; however, an anaerobic glove box equipped with an oxygen-removing catalyst can be used alternatively. In the event of using a glove box, the user should be aware of the prevalent use of H_2 gas as a reductant in these pieces of

equipment, which may provide a contaminating electron donor in enrichment and pure cultures and yield false positives. Airtight vessels that can be flushed with inert gas and include an appropriate catalyst are sufficient for growth on petri plates. When plating cultures and picking colonies in a glove box, the glove box should be equipped with UV light equipment in order to control microbial growth so long as glove box materials are not damaged by UV exposure. In general, when performing anaerobic culturing avoid any technique which risks introducing oxygen into the culture vessel.

2 Chloroxyanion Respiration

The respiration of perchlorate and chlorate is an anaerobic process that requires a (per)chlorate terminal oxidoreductase, a corresponding quinol oxidase, and a chlorite-detoxifying mechanism. In mesophilic perchlorate-reducing bacteria, perchlorate is reduced to chlorate then chlorite by the periplasmic perchlorate reductase (Pcr) (Fig. 2); toxic chlorite is converted to transient benign chloride and molecular oxygen by chlorite dismutase (Cld); the oxygen is then consumed by the same organism [2]. Chlorate-reducing bacteria can only reduce chlorate using chlorate reductase (Clr). The reductase, dismutase, and accessory protein genes for (per)chlorate respiration consist genomic islands or composite transposons, suggesting the metabolism is horizontally transferred [3, 4]. Most isolated (per)chlorate-reducers belong to the phyla Proteobacteria. Archaeal (per)chlorate reduction, only recently discovered, appears to involve periplasmic nitrate reductase (pNar) instead of Pcr or Clr [5, 6]. All three enzymes are molybdenum-dependent members of the DMSO reductase superfamily; therefore all known (per)chlorate-reducing microorganisms require trace amounts of molybdenum [2, 7]. Most bacteria

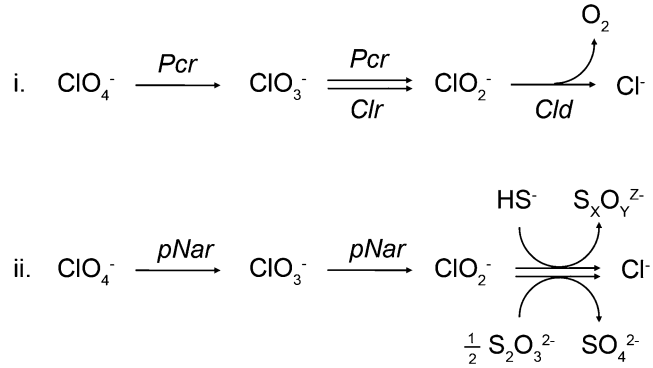

Fig. 2 Alternative pathways for (per)chlorate respiration and associated enzymes

that respire perchlorate also respire nitrate, and almost all (per) chlorate-reducing microorganisms can respire oxygen or tolerate its presence [2]. Despite being less thermodynamically favorable than perchlorate reduction at neutral pH and physiological temperature, nitrate reduction is preferentially utilized in most pure cultures and communities [2, 8]. Perchlorate, chlorate, and nitrate removal can be monitored accurately by ion chromatography.

3 Hydrocarbon Oxidation

Growth by the oxidation of aliphatic and aromatic hydrocarbons has been confirmed for several (per)chlorate-reducing bacteria and can proceed through aerobic, anaerobic, or aerobic-hybrid pathways [8–11]. Hydrocarbon-contaminated sites and bioreactors have provided hydrocarbon-oxidizing isolates of (per)chlorate-reducing bacteria, and though none has been directly isolated from oil reservoirs, metagenomic Cld sequences suggest their presence [12, 13]. (Per)chlorate-reducing bacteria can use a variety of electron donors, most commonly small organic acids, alcohols, and hydrogen, but elemental sulfur, thiosulfate, reduced iron, and reduced humic substances have also been shown to support the growth of some strains.

Growing hydrocarbon-oxidizing organisms presents several challenges owing to the toxicity, volatility, low solubility, and high hydrophobicity of many hydrocarbon compounds and the slow growth rates that accompany the degradation of recalcitrant hydrocarbons [14]. Poorly soluble compounds such as polycyclic aromatic hydrocarbons can be dissolved in a carrier, commonly the inert branched alkane 2,2,4,4,6,8,8-heptamethylnonane (HMN). Most *n*-alkanes and monocyclic aromatic hydrocarbons (such as benzene, toluene, ethylbenzene, and xylenes) do not require a carrier, although consideration must still be given to toxic concentrations. Crude oil, which consists of complex mixtures of aromatic and aliphatic hydrocarbons, can also be supplied. Most hydrocarbons form a separate phase as a film or droplets that will adhere to hydrophobic surfaces such as rubber stoppers; cultures should be incubated in the inverted position at an angle and in large serum bottles (Fig. 3) to maximize surface area for microbial activity and reduce contact between the hydrocarbon phase and the stopper. The concentration of hydrocarbon in aqueous solution is limited by its solubility, so the rate of transfer from hydrocarbon phase to aqueous phase is determined by the rate of hydrocarbon consumption; less-toxic hydrocarbons can be added in excess of their aqueous solubility. Davidova and Suflita [14] suggest adding no more than 10 mg naphthalene per 1 mL HMN carrier. Benzene and toluene, both water soluble, did not inhibit growth at the concentrations tested (150 and 500 μM, respectively). *n*-Alkanes were not found to inhibit microbial growth; note that microbes consume

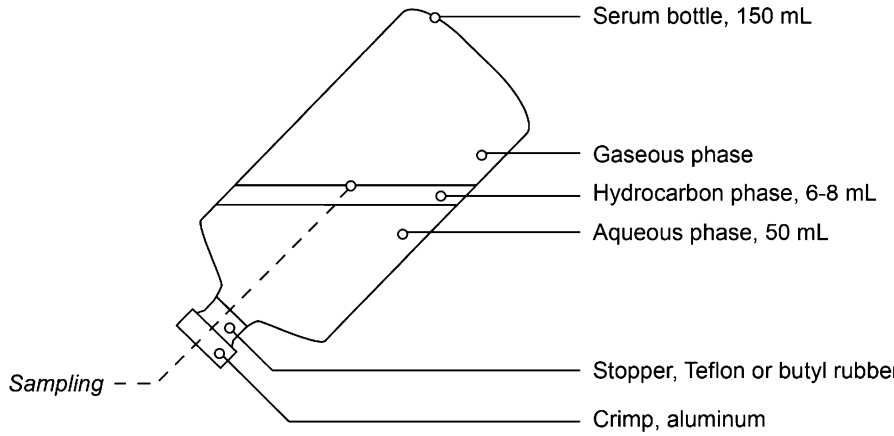

Fig. 3 Demonstration of inverted set-up for hydrocarbon-oxidizing cultures

specific alkanes, so to support diverse growth a mixture of *n*-alkanes with various lengths should be added to enrichments. A water-soluble analogue to the hydrocarbon of interest, such as caproate for *n*-alkanes and 4-chlorobenzoate for aromatic compounds, may be appropriate for improving isolation but could bias enrichment.

Gas chromatography can accurately quantify a variety of hydrocarbons. When a carrier is used, hydrocarbon consumption can be monitored by sampling a small volume (1–2 μL) of carrier phase; otherwise, gas-phase headspace analysis can be used for volatile compounds (BTEX) [9]. Alternatively the hydrocarbon content of sacrificial bottles can be liquid extracted and analyzed. Volatile hydrocarbons can be supplied to agar plates as a gas in an airtight vessel [15] and measured by sampling the headspace.

4 Isolation

(Per)chlorate-reducing microorganisms can be isolated from enrichments or directly from the environment. Isolation of mesophilic (per)chlorate-reducing microorganism from the environment can be accomplished in anaerobic tubes using selective media, serial dilution, and separation in agar. Media-containing perchlorate or chlorate (5 mM) and a non-fermentable electron donor selects for (per)chlorate-reducing microorganisms. Benzoate (1 mM), acetate (10 mM), and hydrogen (101 kPa) are appropriate electron donors, although a carbon source must be added when using hydrogen.

1. Perform serial 1:10 dilutions in anaerobic selective media until the sample concentration ranges from 10^0 to 10^{-9}.
2. Transfer 7 mL of each concentration to 3 mL of sterile molten agar (4% w/v) in an anaerobic tube.

3. Thoroughly mix the sample and agar, and solidify the agar by immersing the inverted tube in ice-cold water.
4. Incubate at the source environment's temperature until individual colonies form.
5. Pick colonies using a Pasteur pipet in an anaerobic glove box, transfer these "plugs" to new media, and monitor growth using microscopy or absorbance at 600-nm wavelength.
6. To ensure isolation, repeat the process a second time and verify isolation by sequencing the 16S rRNA gene using universal primers.

Following isolation, the strain can be characterized by growth using (per)chlorate and varying electron donors and by growth using acetate (if appropriate) and varying electron acceptors. Stocks of (per)chlorate-reducing isolates can be stored at −80°C for long periods of time in 20–30% (v/v) glycerol.

Most probable number (MPN) counts can be used to estimate the number of viable (per)chlorate-reducing cells in a sample [16]. The presence or absence of growth in replicate serial dilutions is used to calculate the likely concentration of cells in the original sample. MPN counts use the same media as for isolation, but adding sodium pyrophosphate (e.g., 3.76 mM) prior to incubation will help separate cells from sediment particles. Incubate for 60 days in the dark to prevent phototrophic growth and determine the presence/absence of growth visually or by microscopy and (per)chlorate consumption by ion chromatography.

5 Enrichment

Enriching environmental samples can improve the success of isolation and offers the opportunity to study simple communities in a laboratory setting. Enrichments for hydrocarbon-oxidizing activity are most successful when the sampled environment has been exposed to hydrocarbons [17]. Samples should be collected in sterile, gas-impermeable containers with no headspace (or flushed with inert gas) and can be briefly stored at 4°C but never frozen. Sample should be mixed in a 1:10 ratio (w/v) with anaerobic media, which can consist of filtered-sterilized water from the sampling site or a laboratory media mimicking environmental salinity, pH, and mineral composition (see: Media). Add perchlorate or chlorate (5 mM) and sub-inhibitory amounts of hydrocarbon as an electron donor and carbon source. If enrichments are prepared in an atmosphere-containing hydrogen, flush the containers with hydrogen-free inert gas prior to incubation. Active enrichments that consume both (per)chlorate and hydrocarbon may be isolated but should be transferred twice beforehand. Because of their slow

growth, transfers of hydrocarbon-oxidizing enrichments are more successful when a concentrated inoculum is generated by centrifugation or, for bacteria that adhere to the hydrocarbon phase, the hydrocarbon is transferred to new media [14]. For identification of the microbial communities in active cultures, collect regular samples in a 0.22-μm filter (remove eukaryotes with a 0.4-μm filter), extract DNA and/or RNA, and store at −20°C for later sequencing.

5.1 Mesophiles, Freshwater

The majority of (per)chlorate-reducing bacteria isolated to date have growth optima at mesophilic temperatures (25–40°C) at circumneutral pH in freshwater ion concentrations [2]. Numerous media have been developed that mimic the low mineral concentration in freshwater, and hydrocarbon-oxidizing (per)chlorate-reducing bacteria have been successfully enriched in these media on 4-chlorobenzoate and benzene from several terrestrial environments, including river sediment, groundwater, and a bioreactor, and their activity has been detected in soil [10, 11, 18, 19].

5.2 Mesophiles, Marine

Only recently have halophilic (per)chlorate-reducing bacteria been enriched and isolated from marine environments under (per)chlorate-selective conditions. Two such strains, both in the genus *Sedimenticola*, have been shown to oxidize aromatic compounds including benzoate and phenylacetate [8]. Though the source environment of *Sedimenticola* sp. strain NSS was contaminated with polycyclic aromatic hydrocarbons, neither strain was enriched using hydrocarbon compounds. However, other marine strains enriched similarly, *Arcobacter* sp. strain CAB and *Shewanella algae* ACDC, did not utilize the aromatic compounds tested [20, 21].

The isolation of extreme halophiles under (per)chlorate-reducing conditions has not yet been accomplished. The archaeon *Haloferax mediterranei*, which contains a narG-type nitrate reductase, can grow by chlorate reduction for several generations if pregrown by nitrate reduction [6], and some *Haloferax* strains can grow by the aerobic oxidation of hydrocarbons, but anaerobic oxidation of hydrocarbons has not been demonstrated for extreme halophiles under any electron accepting conditions [22].

5.3 Thermophiles

Six thermophilic (per)chlorate-reducing microorganisms have recently been characterized. The thermophilic firmicute *Moorella perchloratireducens* was isolated from an underground gas storage tank after enrichment on (per)chlorate and methanol at 55°C [23] and to our knowledge has not been tested for hydrocarbon oxidation. Another thermophilic firmicute, *Moorella humiferrea*, was isolated from a hydrothermal spring after enrichment on lactate and 9,10-anthraquinone-2,6-disulfonate (AQDS) can also use perchlorate as an electron acceptor; no hydrocarbons tested were oxidized in the presence of AQDS

[24]. The hyperthermophilic archaeon *Archaeoglobus fulgidus* strain VC-16 was isolated from a marine hydrothermal vent after enrichment on sulfate and yeast extract at 85°C [25] and later shown to reduce (per)chlorate coupled to the oxidation of lactate [5]. *A. fulgidus* is readily enriched from sites with oil and can degrade fatty acids, *n*-alkanes, and *n*-alkenes [26] but has not yet been shown to couple this metabolism to (per)chlorate reduction. Three other hyperthermophilic organisms, none known to oxidize hydrocarbons, can also respire perchlorate: *Aeropyrum pernix*, *Carboxydothermus hydrogenoformans*, and *Moorella glycerini* strain NMP [27]. For these organisms and *A. fulgidus*, which all lack chlorite dismutase activity, chlorite inhibits growth unless it is removed by abiotic reactions with reduced sulfur compounds supplied in the medium or generated in vivo by sulfate reduction [12, 13, 27]. Therefore supplying reduced sulfur compounds in media might improve the enrichment and isolation of organisms that lack chlorite dismutase. Otherwise, the media and methodology for enriching and isolating (hyper)thermophiles are identical to that for mesophiles, but molten agar must be permitted to gel at low temperature prior to incubation at high temperatures and consideration must be given to increased pressure at high temperatures.

6 Media

The below media instructions are described without any electron acceptor or electron donor (except in trace amounts) such that a 1-mL inoculation yields a 10-mL final volume in each anaerobic tube. The chosen donor and acceptor for your media should be added before autoclaving if possible. When making these media, add components in the order listed and allow each component to dissolve before adding the next component. These media are bicarbonate-buffered and therefore require HCO_3^- in solution and CO_2 (20% by volume) in the headspace to maintain the appropriate pH; other buffering systems may be more appropriate for particular experiments.

6.1 Mineral Mix

Component	Amount (g L^{-1})
Nitrilotriacetic acid (NTA)	1.5
$MgSO_4$	3.0
$MnSO_4 \cdot H_2O$	0.5
NaCl	1.0
$FeSO_4 \cdot 7H_2O$	0.1

(continued)

(continued)

Component	Amount (g L^{-1})
$CaCl_2 \cdot 2H_2O$	0.1
$CoCl_2 \cdot 6H_2O$	0.1
ZnCl	0.13
$CuSO_4$	0.01
$AlK(SO_4)_2 \cdot 12H_2O$	0.01
H_3BO_2	0.01
Na_2MoO_4	0.025
$NiCl_2 \cdot 6H_2O$	0.024
$Na_2WO_4 \cdot 2H_2O$	0.025

6.2 Vitamin Mix

Component	Amount (mg L^{-1})
Biotin	2.0
Folic acid	2.0
Pyridoxine HCl	10.0
Riboflavin	5.0
Thiamin	5.0
Nicotinic acid	5.0
Pantothenic acid	5.0
Cyanocobalamin (B_{12})	0.1
P-aminobenzoic acid	5.0
Thioctic acid	5.0

6.3 Freshwater Medium

Component	Amount (g L^{-1} or mL L^{-1})
NH_4Cl	0.25 g
NaH_2PO_4	0.60 g
KCl	0.1 g
$NaHCO_3$	2.5 g
Vitamin mix	10 mL
Mineral mix	10 mL

Instructions:

1. Combine all medium components in a large flask.
2. Bring medium to a boil and cool to room temperature while sparging with a gas mixture of 80% N_2 and 20% CO_2 and adjust to desired pH (generally 6.8).
3. Under a flow of N_2/CO_2 gas, distribute 9 mL into each tube. Seal and crimp.
4. Autoclave for 20 min above 120°C.

6.4 Marine Medium

Component	Amount (g L^{-1} or mL L^{-1})
NaCl	30 g
KCl	0.67 g
$NaHCO_3$	2.5 g
Vitamin mix	10 mL
Mineral mix	10 mL
Salt solution A	20 mL

Salt Solution A

Component	Amount (g L^{-1})
NaCl	40
NH_4Cl	50
KCl	5
KH_2PO_4	5
$MgSO_4 \cdot 7H_2O$	10
$CaCl_2 \cdot 2H_2O$	1

Instructions

1. Combine all medium components in a large flask.
2. Bring medium to a boil and cool to room temperature while sparging with a gas mixture of 80% N_2 and 20% CO_2 and adjust to desired pH (generally 7.8).
3. Under a flow of N_2/CO_2 gas, distribute 8 mL into each tube. Seal and crimp.
4. Autoclave for 20 min above 120°C.
5. Add presterilized anoxic 0.5 mL $MgCl_2 \cdot 6H_2O$ (2.12 g L^{-1} stock) and 0.5 mL $CaCl_2 \cdot 2H_2O$ (0.304 g L^{-1} stock) to each tube.

Salinity (total mass of dissolved ions per liter) can be adjusted by varying the amount NaCl added.

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Cultivation of Methanogens

Taiki Katayama and Yoichi Kamagata

Abstract

Methanogens are methane-producing archaea that thrive only under strictly anaerobic conditions where electron acceptors (sulfate, nitrate, oxidized forms of metals, etc.) other than CO_2 are depleted. Because of their nature, not only anoxic but highly reduced conditions with a redox potential as low as −300 mV must be retained during all processes of cultivation by the following essential steps: flushing out air from medium with O_2-free gases, sealing with gas-tight closures, and supplying reducing agents. The medium used for methanogens should have CO_2/bicarbonate/carbonate buffering system and is composed of minerals, trace metals, vitamins, and methanogenic substrates. Based on the limited number of substrates known, unless otherwise specified, H_2, acetate, or methanol is generally supplied to the medium as energy source. The protocol described here introduces the basic techniques for cultivation (pure culture and enrichment) of methanogens including medium preparation, sample inoculation, and colony isolation using Hungate roll tube, deep agar slant, and recently developed six-well plate method and coculture methods. The protocols are simple and easy and do not require an anaerobic box, the most costly equipment.

Keywords: Anaerobic cultivation, Coculture, Hungate technique, Methanogen, Methanogenesis, Roll tube technique, Six-well plate technique, Syntroph

1 Introduction

Methanogens are a diverse group of archaea that produce methane from very limited substrates such as H_2/CO_2, methylated compounds, and/or acetate. Methane production by methanogens, which is called methanogenesis, occurs as a terminal step of anaerobic degradation of organic matters where electron acceptors other than CO_2, such as NO_3^-, SO_4^{2-}, or oxidized forms of metals (e.g., Fe^{3+}), are depleted. Methanogens are extremely sensitive to oxygen and require a redox potential below −200 to −300 mV. Therefore, anoxic and reduced conditions are required during cultivation. This can be achieved by the following steps: expelling air from cultivation medium with O_2-free gases, sealing the culture vials or tubes with gas-tight butyl rubber stoppers and aluminum crimps (*see* **Note 1**), and supplying reducing agents (*see* **Note 2**) and a redox indicator dye (*see* **Note 3**) to keep and monitor reduced

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conditions. In the end, the gas phase would be replaced with H_2/CO_2, when hydrogenotrophic methanogens are cultivated. These techniques are essentially based upon those developed by Hungate [1] and Balch et al. [2], the former of which is hereafter referred to as the Hungate roll tube method.

When one would like to isolate methanogens from microbial communities in natural or anthropogenic environments, one would not have to care so seriously about the anaerobicity of the prepared media as the communities usually harbor facultative anaerobes that could immediately consume O_2 (though marine and oligotrophic environments are not always the case). In the primary enrichment, even reducing agents may not be needed. However, as methanogens become more dominant after consecutive passages of the enrichment, facultative anaerobes irrelevant to competition over the substrate become fewer; thus, the media need more strictly anaerobic conditions.

Methanogens can be divided into three groups based on their substrate utilization: hydrogenotrophic (reducing CO_2 to methane with H_2 or formate), acetoclastic (cleaving C–C bond in acetate to produce methane from the methyl moiety), and methylotrophic methanogens (that produce methane from methyl groups by using electrons provided from oxidation of other methyl groups). Some hydrogenotrophic methanogens can also utilize primary and secondary alcohols, such as ethanol and 2-propanol, as electron donors for CO_2 reduction to methane. These substrates are added to media before or after the media are autoclaved (*see* **Note 4**). Some hydrogenotrophic methanogens require acetate as a carbon source. Requirements of growth factors, such as yeast extract, peptone, vitamins, and/or coenzyme M, are frequently reported, but this fully depends on methanogenic species or even strains. Physiological characteristics of methanogens are so diverse from psychrophiles to hyperthermophiles, acidophiles to alkaliphiles, and non-halotolerant to extreme halophilic species that media or growth conditions should be adjusted to respective trait. Extremophilic methanogens are exemplified by several strains like *Methanogenium frigidum* that was isolated from the Ace Lake, Antarctica, and that preferably grows at 15°C [3] and *Methanopyrus kandleri* that was isolated from deep subseafloor hydrothermal sediment and grows as high as 110°C [4]. It is thus evident that medium compositions and growth conditions vary depending on the methanogenic species.

In isolating methanogens from environmental samples, the substrate used is the crucial factor selecting the target methanogens. To capture as many species of methanogens as possible in the enrichment procedure, all possible substrates can be added into one medium or medium containing each methanogenic substrate should be prepared. Enrichment often selects some specific methanogens that are less dominant in the environment where a diverse and/or other dominant methanogens of researchers' interest are

present. Researchers have long been suffering from this problem that still remains unsolved. One of the typical reasons is that minor species outcompete major species because of their growth rate under the artificial laboratory conditions.

This chapter introduces techniques for cultivation and isolation of methanogens that include medium preparation, inoculation, and colony isolation from liquid and solid media using Hungate roll tube, deep agar slant, and recently developed six-well plate method [5] based on dilution-to-extinction techniques. In addition, coculture method, a novel and elaborate technique for isolating elusive methanogens from environments [6, 7], is also described. Note that all protocols described here do not need to use an anaerobic chamber, the most costly and cumbersome equipment for anaerobic culture. For details of the procedures using an anaerobic chamber, readers should refer to the literatures by Sowers and Noll [8] and recent protocol by Speers et al. [9] and Wolfe [10]. The methods described in this chapter work well unless readers would like to purify oxygen-labile enzymes or perform genetic manipulations.

2 Materials

2.1 Liquid Culture

1. Gassing manifold: Dexygenized Gas Pressure & Replace Injector type GR-8 (Sanshin Industrial, Co. Ltd. (http://www.sanshinkogyo.co.jp/e/pdt_anaerobic_gr8.html)) or functionally equivalent apparatus.
2. Kanamycin (Sigma–Aldrich (https://www.sigmaaldrich.com)) or equivalent.
3. Vancomycin (Sigma–Aldrich (https://www.sigmaaldrich.com)) or equivalent.

2.2 Hungate Roll Tube Method

1. Noble agar (Becton–Dickinson (http://www.bd.com/)) or equivalent. The type of agar sometimes influences the growth of microorganisms.

2.3 Six-Well Plating Method

1. Gellan gum (Wako Pure Chemical Industries, Ltd. (http://www.wako-chem.co.jp/english/)) or equivalent.
2. Nylon bag (W-Zipper Pouch-Bag, Mitsubishi Gas Chemical Company (http://www.mgc.co.jp/eng/index.html)).
3. Catalyst sachet (AnaeroPack-Anaero, Mitsubishi Gas Chemical Company (http://www.mgc.co.jp/eng/index.html)).

These may be purchased from any supplier.

2.4 Coculture Method

1. The type strain of *Syntrophobacter fumaroxidans* strain DSM 10017^T is available in DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, in Germany (http://www.dsmz.de/home.html). Substrates for growth: sodium propionate and monosodium fumarate. As *S. fumaroxidans* is a mesophilic syntrophic propionate-oxidizing H_2-producing bacterium [11], one should choose an alternative to this bacterium if one would like to attempt to cultivate and isolate high-temperature methanogens or methanogens that are not suitable to attempt cultivation with *S. fumaroxidans* in terms of their physiological traits.

2.5 Solutions for W and WS Media

1. Mineral solution (tenfold concentration) (per 1 L): 5.35 g NH_4Cl (100 mM), 1.36 g KH_2PO_4 (10 mM), 2.04 g $MgCl_2 \cdot 6H_2O$ (10 mM), and 1.47 g $CaCl_2 \cdot 2H_2O$ (10 mM). Autoclave and store at 4°C.
2. Sodium chloride (used for WS medium; *see* Subheading 3.1).
3. Sodium bicarbonate.
4. Trace elements solution (1,000-fold concentration) (per 1 L): 12.8 g nitrilotriacetic acid (NTA), 1.27 g $FeCl_2$ (10 mM), 0.13 g $CoCl_2$ (1 mM), 0.198 g $MoCl_2 \cdot 4H_2O$ (1 mM), 0.136 g $ZnCl_2$ (1 mM), 6.2 mg HBO_3 (0.1 mM), 13 mg $NiCl_2$ (0.1 mM), 13.3 mg $AlCl_3$ (0.1 mM), 24.2 mg $Na_2MoO_4 \cdot 2H_2O$ (0.1 mM), 1.3 mg $CuCl_2$ (0.01 mM), 1.7 mg Na_2SeO_3 (0.01 mM), and 3.3 mg $Na_2WO_4 \cdot H_2O$ (0.01 mM). Autoclave and store at 4°C. Dissolve NTA first in 200 mL of Milli-Q water and adjust pH to 6.5 with KOH. Then, dissolve mineral salts one by one. Dilute the solution with Milli-Q water up to 1 L.
5. Resazurin solution (1,000-fold concentration) (per 1 L): 1 g resazurin. Store at 4°C.
6. Vitamin solution (1,000-fold concentration) (per 1 L): 5 mg biotin, 5 mg *p*-aminobenzoic acid, 5 mg pantothenate Ca salt, 10 mg pyridoxine HCl, 5 mg nicotinic acid, 5 mg thiamine HCl $\cdot 2H_2O$, 5 mg lipoic acid (thioctic acid), 5 mg folic acid, 5 mg B_{12}, and 5 mg riboflavin. Store at 4°C.
7. 1.2% (w/v) Na_2S or L-cysteine solution. Dispense 30 mL of Milli-Q water to a 50-mL serum vial. Purge the water with N_2 gas for 10 min (*see* **Note 5**). Dissolve 0.36 g of $Na_2S \cdot 9H_2O$ and/or L-cysteine HCl and then seal with a butyl rubber and an aluminum crimp. Store at 4°C. Filter-sterilize when added to the culture medium.
8. 100 mM titanium (III) citrate solution: it is prepared according to the method described by Zehnder and Wurhmann [12]. In brief, Add 2 mL of a 15% titanium (III) chloride solution (*see* **Note 6**) to 20 mL of 0.2 M sodium citrate solution in a 50-mL

serum vial under an oxygen-free atmosphere. Neutralize it with a saturated sodium carbonate solution under an oxygen-free atmosphere. Seal with a butyl rubber and an aluminum crimp. Store at 4°C. Filter-sterilize when added to the culture medium. Titanium citrate solution is a powerful reducing agent sometimes used for cultivating methanogens and other strictly anaerobic organisms. However, as shown in **Note 7**, there are several critical drawbacks for its use.

9. 50 mM titanium (III) nitrilotriacetate solution: it is prepared according to the method described by Moench and Zeikus [13]. In brief, Add 4.8 mL of 0.5 M sodium nitrilotriacetate (NTA) solution to 7.2 mL of 1.0 M Tris/HCl buffer (pH 8.0) in a 50-mL serum vial under an oxygen-free atmosphere. Add 0.55 mL of a 15% titanium (III) chloride solution (*see* **Note 6**). Seal with a butyl rubber and an aluminum crimp. Store at 4°C. Filter-sterilize when added to the culture medium.
10. 0.2% (w/v) kanamycin or vancomycin solution. Store at 4°C. Filter-sterilize when added to the culture medium.

3 Methods

The cultivation process of methanogens consists of medium preparation, inoculation, incubation, and verification of the growth of the methanogen. Cultivation of pure methanogens is straightforward, but it needs some modifications when enrichment and isolation are performed as described elsewhere. During the medium preparation, oxygen must be removed from liquid solutions of medium components by purging with O_2-free gas. An oxygen scrubber and gassing manifold are necessary to remove traces of oxygen from commercially available compressed gases and to dispense anoxic gases, respectively. A multichannel gassing manifold combined with an oxygen scrubber allows preparing large numbers of anaerobic media and is commercially available from several manufacturers (Fig. 1). To prevent the co-growth of fast-growing bacteria that compete for methanogenic substrates (such as acetogens), bacteria-specific antibiotics, such as β-lactam, aminoglycoside, and glycopeptide antibiotics, are sometimes added to the culture. During or after cultivation, the presence of methanogens in a culture can be determined by gas chromatographic analysis of methane in the headspace (*see* **Note 8**) or epifluorescence microscopic analysis of cells (*see* **Note 9**). Due to a distinct coenzyme F_{420}, an electron carrier specifically present in methanogen, cells autofluoresce [14] by excitation wavelength of around 400 nm, which is useful to observe methanogenic cells.

To isolate a methanogen in pure culture from environmental samples, either Hungate roll tube or deep agar slant can be used.

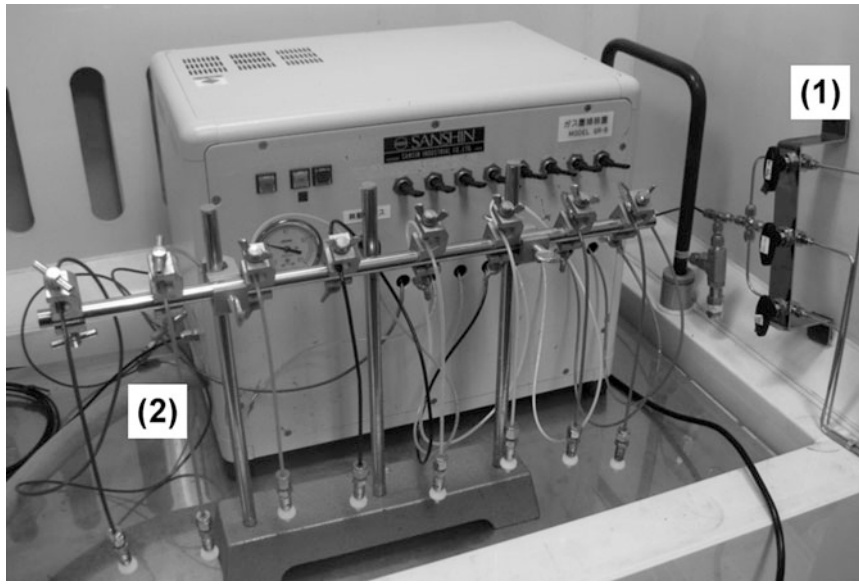


Fig. 1 Gassing manifold (Dexygenized Gas Pressure & Replace Injector type GR-8, Sanshin Industrial, Co. Ltd.). High-pressure gas cylinders are connected to a reactor containing a copper-based catalyst. The manifold is equipped with three-way valve [1] to pass three different gases, N_2, N_2/CO_2, and H_2/CO_2, through eight cannulas [2] after removing O_2 by passing the gas through the heated copper reactor. The outlet of cannula can connect with disposable syringe filter, needle, etc. as usage

Besides these two methods, recently developed six-well plate method can be also applied [5]. Dilution-to-extinction series of liquid culturing can be used to isolate methanogens that do not form visible colonies on solid media. The advantages and disadvantages of these solid cultures are described below. If the target methanogens are extremely oxygen sensitive, the use of anaerobic chamber is recommended.

3.1 Liquid Culture

The basal medium described in this chapter is based on that of Widdel and Pfennig [15], designated here as W medium. The culture medium used for methanogens from marine environments (designated here as WS medium) is also described. In W or WS medium, $NaHCO_3$ and CO_2 are added to make a buffer of CO_2–HCO_3^-–CO_3^{2-} for maintaining pH of medium around neutral. H_2/CO_2 gas (80:20, v/v), acetate (10–20 mM), or methanol (10–20 mM) is generally used for the cultivation of hydrogenotrophic, acetoclastic, or methylotrophic methanogens, respectively. The methane production in batch culture containing soluble methanogenic substrates generates overpressure, which sometimes causes explosion of a glass container. To avoid this risk, pressure culture tubes are usually used (*see* **Notes 10** and **11**). In addition, liquid medium filling only up to 25% of the vials used is recommended (*see* **Note 12**). Here, the preparation of liquid medium and

inoculation (pure culture or natural community samples) are described.

1. Dispense 500 mL of Milli-Q water to a 1-L beaker. Dissolve 100 mL of mineral solution and 1 mL of trace elements, resazurin, and vitamin solutions (*see* Subheading 2.5).
2. Dissolve 2.52 g/L of $NaHCO_3$ (30 mM) (*see* **Note 13**) and dilute up to 1 L (*see* **Note 14**).
3. For WS medium, final concentration of $MgCl_2 \cdot 6H_2O$ should increase to 15–50 mM and NaCl to 350–400 mM.
4. Purge with N_2/CO_2 gas (80:20, v/v) for 20 min (*see* **Note 5**).
5. Dispense 20 mL of the solution to a 50-mL serum vial and purge with N_2/CO_2 gas again for 5 min.
6. Seal with a butyl rubber and an aluminum crimp, and autoclave at 121°C for 15 min.
7. Cool down autoclaved vials.
8. Sterilize the butyl rubber septum by wiping with 70–75% ethanol followed by flaming (*see* **Note 15**).
9. Add 0.5–0.8 mL each of 1.2% Na_2S and L-cysteine solutions (final conc. c.a. 0.03–0.05%), 0.04–0.1 mL of 100 mM titanium citrate solution (final conc. 0.2–0.5 mM), or 0.4 mL of 50 mM titanium NTA solution (final conc. 1 mM) (*see* Subheading 2.5) per vial (*see* **Note 16**).
10. Add sterilized methanogenic substrate solutions or gaseous substrate (H_2/CO_2, 80:20, v/v).
11. Add 0.5 mL each of filter-sterilized 0.2% kanamycin and vancomycin solutions and/or other bacterial antibiotics if needed.
12. Inoculate what is to be cultivated with sterile needle syringe (*see* **Note 17**). If environmental samples are inoculated to enrich and isolate methanogens, the way of inoculation should be changed. For instance, if the sample is soil, mud, or sediment, a syringe may not be used for inoculation. If so, remove the aluminum crimp and butyl rubber septum, add the sample by pipette or appropriate means, seal again with a sterilized butyl rubber and an aluminum crimp, and replace the headspace gas with N_2/CO_2.

3.2 Dilution-to-Extinction Method

Dilution-to-extinction method, whereby samples are serially diluted with culture medium down to single cells, has been used not only to estimate the number of viable microbial cells, but also to physically reduce the number and diversity of microbial species within samples. Since some methanogens do not form visible colonies in solid media, this method can be used to isolate such methanogenic species. As already mentioned, the addition of bacterial-specific antibiotics would be effective for this purpose. In addition,

dilution-to-extinction series are usually prepared before their culture in solid media (*see* Subheadings 3.3, 3.4, and 3.5) to make a certain number of colonies that are suitable for isolation. The dilution rate depends on microbial population density in the sample.

1. Prepare the 9 mL of sterilized and reduced W or WS medium containing methanogenic substrates (*see* **Note 18**) in a 20-mL test tube (for medium preparation, *see* Subheading 3.1).
2. Inoculate 1 mL of a sample with needle syringe. Invert to mix well.
3. Transfer 1 mL of tenfold diluted sample to fresh culture (*see* **Note 19**).
4. Repeat the serial dilution to fresh culture until it reaches to desired dilution rate.

3.3 Hungate Roll Tube

The Hungate roll tube [1] is the most widely used method for isolation of strict anaerobes. The inoculated agar medium in a tube is rotated in flowing cold water using a mechanical device or rotated on ice manually to solidify the medium on the inside surface of the tube, making the vertical cavity within the tube. Cultivation at higher temperature (>55°C) is not suitable due to the breakdown of a solidified agar (*see* **Note 20**). Longer incubation time results in separation of more water from agar, which eventually dries a gel. The visibility of colonies is better than that in deep agar slant. We usually use a 50-mL serum vial instead of a test tube. In this case, it should be called roll "vial" instead of roll tube, but the accessibility to colonies is far better because the cavity space is larger than that of a test tube. The wide surface area of agar allows wide contact to gas phase thus faster growth when hydrogenotrophic methanogens are cultured with H_2/CO_2. Also, it allows precise colony picking when isolating methanogens. The procedure presented below uses a vial.

1. Prepare the 5–20 mL of sterilized and reduced W or WS medium containing methanogenic substrates (*see* **Note 18**) in a 50-mL serum vial (*see* Subheading 3.1).
2. Wash Noble agar (*see* **Note 21**) with Milli-Q water twice (*see* **Note 22**).
3. Suspend the washed agar in Milli-Q water to make 4% (w/v) concentration.
4. Dispense 5 mL of the agar suspension into a 50-mL vial, sparge it with N_2/CO_2 gas, and seal the vial with a butyl rubber and an aluminum crimp.
5. Autoclave at 121°C for 10 min. Keep both molten agar and medium at 55°C until use.
6. Inject 5 mL of medium into the vial containing molten agar using a sterile, anoxic needle syringe (20–22 gauge) and mix

well (*see* **Notes 23** and **24**). Keep it again at 55°C until the next step.

7. Inoculate 0.1–1.0 mL of serial dilution of the cell suspension (*see* **Note 25**) using a sterile needle syringe (*see* **Note 17**). Mix well (*see* **Note 26**).
8. Lay the vial on its side and rotate it on ice rapidly to form an agar roll (*see* **Note 27**).
9. Replace the gas phase in each vial with N_2/CO_2 gas and incubate it in an upright position.
10. Prior to colony isolation, prepare colony pickers by bending the tips of Pasteur pipettes at a 90° angle using a Bunsen burner. Sterilize pipettes by dry-heat sterilization at 180°C for 3 h (*see* **Note 28**).
11. Purge the pipette with N_2 or N_2/CO_2 gas (*see* **Note 29**).
12. Decap the aluminum crimp and butyl rubber from the roll tube and immediately position the pipette tip into the roll tube. Press the tip into the agar and remove a plug of agar containing a colony (*see* **Note 30**).
13. Transfer the picked colony into fresh culture medium (*see* **Note 31**).
14. Reseal the tube and replace gas phase with O_2-free gas of both roll tube and fresh culture.
15. Repeat all procedures twice or three times to obtain pure culture.

3.4 Deep Agar Slant Method

A slant solid agar medium is also used for colony isolation of methanogens (Fig. 2). Compared with the roll tube, thicker agar layer delays the oxidation and drying of culture medium. Therefore, this method is suitable for a long period of incubation. The separated water from an agar is pooled into the bottom of the slant (Fig. 2a), which prevents the contaminations during colony manipulation. While at the same time, the thick agar has sometimes difficulty in colony observation. Picking a colony from the deeper layer using a needle involves taking a risk of the contamination with the nontargeting colonies present in the shallow layer.

1. Prepare the 15–20 mL of sterilized and reduced W or WS medium containing methanogenic substrates in a 50-mL serum vial (*see* Subheading 3.1).
2. Prepare the sterilized 5 mL of 4% agar suspension (*see* Subheading 3.3). Keep it at 55°C until use.
3. Inject 15 mL of medium into molten agar using a sterile needle syringe and mix well (*see* Subheading 3.3). Keep it again at 55°C until the next step.
4. Inoculate 0.1–1.0 mL of serial dilution of the cell suspension using a sterile, anoxic needle syringe.

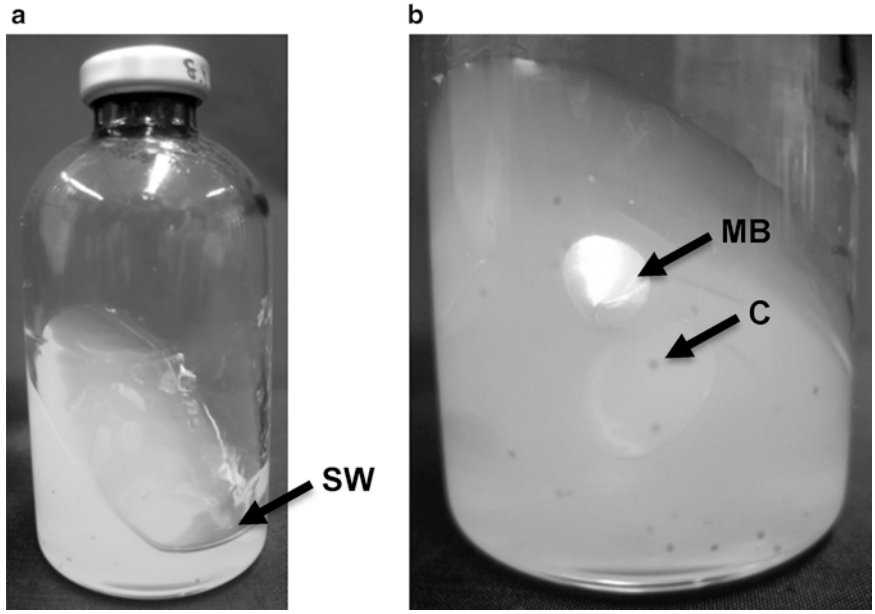
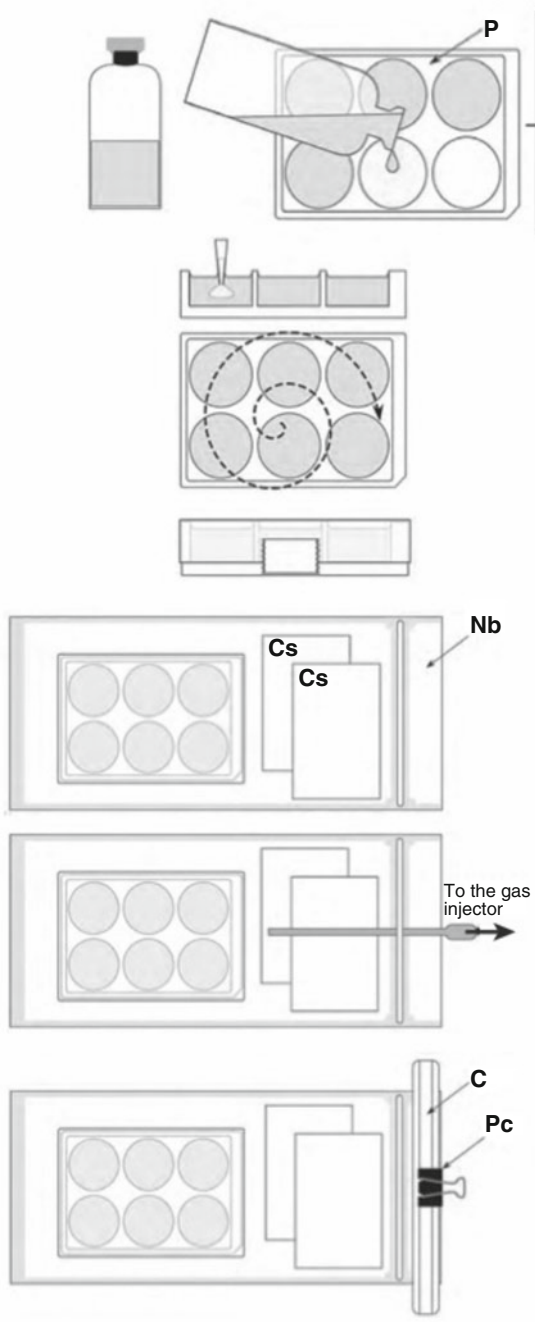


Fig. 2 Deep agar slant culture of methylotrophic methanogen, *Methanohalophilus levihalophilus* strain GTA13^T [16], with trimethylamine as methanogenic substrates. After incubation, separated water (SW) is pooled into the bottom of the slant (**a**) and methane gas bubbles (MB) together with colonies (C) are visibly observed (**b**)

5. Place the vial at a slant at room temperature until the agar is solidified and incubate.
6. After incubation, decap the aluminum crimp and butyl rubber, and insert a sterile needle syringe (*see* **Note 17**) to pick the targeted colony (*see* **Note 32**). Aspirate the colony by pulling a plunger little by little.
7. Transfer the picked colony into a fresh, desired growth medium (*see* **Note 33**).
8. Repeat all procedures twice or three times to obtain pure culture.

3.5 Six-Well Plating Method

Nakamura et al. [5] developed a simple, less laborious method to cultivate and isolate strict anaerobes using a disposable six-well plate and an anaerobic plastic pack (Fig. 3). They evaluated that the anaerobic and reduced conditions were well maintained during cultivation of methanogens as well as the roll tube method. Another advantage of this method is the ease of colony manipulation. Stereomicroscopy would be effective for observation of small colony and manipulation from the plate, which would be very difficult in Hungate roll tube or deep agar culture method. The procedure described below uses a gellan gum as a solidifying agent (*see* **Note 34**), as was done by in Nakamura et al. [5]. The way of



Step 1 Prepare gellan gum medium

Step 2 Stored at 45 or 55°C until use

Step 3

Dispense the molten medium into wells of 6-well plate (P) on a clean bench

Step 4

Inoculate culture dilutions into medium with a dispenser, and rotate plate flatly to suspend inoculated cultures homogeneously in medium

Step 5

Tape lid with the plate

Step 6

Put the plate and two catalyst sachets (Cs) into nylon bag (Nb)

Step 7

Insert needle connected to Deoxidized Gas Pressure Injector equipped with a degassing pump and exchange interior atmosphere with O2-free gas (N_2/CO_2 or H_2/CO_2)

Step 8

Zip the bag completely and transfer to incubator; if filled with H_2/CO_2, secure top of the bag with a clip (C) and a paper clip (Pc)

Fig. 3 Schematic illustration of brief procedure of six-well plate method with aerobic inoculation. The figure is reproduced with slight modifications from the Nakamura et al. [5]

colony manipulation is the same as that described in deep agar slant method (*see* Subheading 3.4).

1. Prepare 80 mL of sterilized and reduced W medium containing gellan gum (0.5–0.6%, w/v) (*see* **Note 35**) in a 100-mL serum vial (*see* Subheading 3.1). Keep it at 45°C or 55°C until use (*see* **Note 36**).
2. Dispense the molten medium into wells of six-well plate aerobically on a clean bench (*see* **Note 37**).
3. Inoculate the serial dilution of cell suspensions immediately into the plate with a dispenser.
4. Rotate the plate flatly and carefully to mix thoroughly before solidifying the medium (*see* **Note 38**).
5. Put the covered plate into a nylon bag together with two catalyst sachets that absorbs oxygen.
6. Insert a flat-end needle that is connected to a gas-exchange machine with a vacuum pump. Exchange the interior atmosphere with N_2/CO_2 gas for three times (*see* **Note 39**).
7. Zip the bag, further seal it with a clip and incubate.
8. Picking colonies and subsequent procedures are the same as the Hungate roll tube method.

3.6 Coculture Method

The addition of H_2/CO_2 gas (around 10^4–10^5 Pa of H_2 partial pressure) is a canonical method for cultivation of hydrogenotrophic methanogens. However, in natural environments besides several geological settings such as hydrothermal vents in a sea floor, in situ H_2 concentrations may be much lower (some 10 Pa) than those in laboratory cultivation conditions. Sakai el al. [6, 7] applied a coculture method based upon interspecies H_2 transfer, i.e., H_2-producing syntrophic oxidation of volatile fatty acids coupled to hydrogenotrophic methanogenesis. In this process, low concentrations of H_2 are continuously provided from a syntroph to a methanogen. Using propionate-oxidizing bacterium *Syntrophobacter fumaroxidans* [11] and propionate as syntrophic substrate, they succeeded in isolating a hydrogenotrophic methanogen *Methanocella paludicola* [17], formerly known as a member of Rice Cluster I, which is an abundant and ubiquitous group in rice paddy soils but eluded isolation for a long time. If we can have a device that continuously feeds very low concentrations of H_2 by using an H_2-generating or H_2-providing apparatus under strictly anaerobic conditions, that should work for isolating novel methanogens with high affinity to H_2. But those cultivation devices are not currently available. Coculture method is therefore the one that harnesses microbial H_2 production and transfer from one organism to the other. The important point is that H_2 produced by syntroph has to be consumed immediately by hydrogenotrophic microorganisms

because of thermodynamic reasons. Under standard conditions, propionate oxidation, for instance, is endergonic as follows:

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3H_2 \text{ ---- } \Delta G^{0'} = +76.4 \text{ kJ} \quad \text{(A)}$$

If the reaction (A) is coupled with hydrogenotrophic methanogenic reaction (B), whole reactions (A+B) become endergonic:

$$3H_2 + 3/4HCO_3^- + 3/4H^+ \rightarrow 3/4CH_4 + 9/4H_2O \text{ ---- } \Delta G^{0'} = -101.7 \text{ kJ} \quad \text{(B)}$$

$$CH_3CH_2COO^- + 3/4H_2O \rightarrow 3/4CH_4 + CH_3COO^- + 1/4H^+ + 1/4HCO_3^- \text{ ---- } \Delta G^{0'} = -25.6 \text{ kJ} \quad \text{(A + B)}$$

This implies that propionate-oxidizing H_2-producing syntroph can never overproduce H_2 due to the thermodynamic constraint thus can constantly provide low concentration of H_2 to methanogens. Therefore, this coculture method is useful to enrich methanogens that have a higher affinity for H_2 and may be well adapted to the low-H_2 habitats. By using this coculture method, Imachi et al. [18] and Tian et al. [19] isolated novel hydrogenotrophic methanogens.

When applying this method, the grown cells of substrate-oxidizing H_2-producing syntroph must be prepared beforehand. It is important to select H_2-providing syntrophs depending upon the target methanogens to be isolated. If one would like to isolate thermophilic methanogens, the H_2-producing syntroph should be also thermophilic growing at the same range of growth temperature as the target methanogens. Here we describe an example of coculture method for enrichment and isolation of mesophilic hydrogenotrophic methanogens from an environmental sample using *S. fumaroxidans* as a syntrophic H_2-providing partner.

1. Culture *S. fumaroxidans* alone in a liquid W medium containing 20 mM propionate and fumarate under an N_2/CO_2 atmosphere at 37°C (*see* **Note 40**).
2. Harvest the *S. fumaroxidans* cells at the exponential growth phase by centrifugation, resuspend the cells in a small volume of fresh W basal medium, and inoculate into a fresh liquid W medium containing 20 mM propionate (but in the absence of fumarate).
3. Add an environmental sample to the medium containing pregrown *S. fumaroxidans* cells with a serial dilution and incubate.
4. Once propionate is degraded concomitantly with methane production, methanogens are most likely to grow on H_2 produced by *S. fumaroxidans*.
5. Transfer the culture to the fresh medium containing the *S. fumaroxidans* cells and propionate.

6. Check the growth of the targeted methanogens by using epifluorescence microscopy to detect F420 containing cells, fluorescence in situ hybridization targeting specific methanogens, or PCR, cloning, and sequencing of 16S rRNA genes or the gene encoding methyl-coenzyme M reductase (*mcrA*).
7. If the target methanogens become dominant, make dilution culture. Cultures receiving the highest dilution that produces methane can be subject to colony isolation by using Hungate roll tube technique or other means as described above in the presence of H_2/CO_2, the direct substrate for the target methanogen.
8. Repeat isolation.

4 Notes

1. For methanogenic culture, the septum or stopper made of butyl rubber must be use to prevent permeation of air into the medium vessel and to allow the repeated puncturing of the septum with needles. Hungate-type and Balch-type tubes and serum vials are commercially available. The Hungate-type tube is closed with a butyl rubber septum and a screw cap, while Balch-type tube or serum vial is sealed with an aluminum crimp to hold a butyl rubber stopper. A special crimper and decapper are necessary to seal and remove the aluminum crimp, respectively.
2. L-cysteine HCl, Na_2S, or titanium (III) citrate is generally used as a reducing agent for cultivation of methanogens. Usually L-cysteine HCl is added to the medium together with Na_2S to maintain highly reduced conditions that allow methanogens to grow. Which reducing agent with what concentrations to be used varies depending on methanogenic species. The hydrogenotrophic *Methanosphaerula palustris* [20] and *Methanoregula boonei* [21] are reported to be sensitive to millimolar concentrations of sulfide.
3. Resazurin is the major redox indicator used for anaerobic cultivation. A medium becomes dark-blue color by the addition of resazurin. After autoclaving, resazurin is irreversibly reduced to resorufin, changing the medium color to be pink at neutral pH (under alkaline conditions, the color may change to blue). As soon as reducing agents are added, resorufin is reduced to hydroresorufin that exhibits colorless. Since this reaction is reversible, the medium color returns to be pink when a redox potential increases.
4. Alternatively, other indirect methanogenic substrates, such as volatile fatty acids (e.g., propionate and butyrate), primary

alcohols (e.g., ethanol), and aromatic compounds (e.g., benzoate), are used to enrich and isolate fastidious methanogens from environments [7] (*see* Subheading 3.6).

5. To remove oxygen from Milli-Q water efficiently, boil the water and then purge with N_2 gas by cooling. Due to its complexity, we skip this heat-and-cool process unless the volume of culture medium in a vial is large (>100 mL per vial). Alternatively, a vacuum-vortex technique developed by Wolfe and Metcalf [22] can be used to prepare anoxic solution without heating and cooling steps. In this method, anoxic conditions in a vial can be made by three alternate cycles of strong vortexing under high negative pressure followed by addition of N_2 gas.
6. Special care is required when handling, because titanium (III) chloride is flammable and an acute toxic substance that irritates the skin and eyes.
7. Some kinds of precipitates often appear in the culture supplemented with titanium (III) citrate that makes it difficult to check the growth by turbidity. Also, despite of its high reducing power, it is relatively readily oxidized and thus may be difficult to maintain the redox potential for a long period of incubation compared with Na_2S solution. This may be a critical drawback for cultivating slow-growing methanogens.
8. In the methanogenic culture using a solid medium that contains methanogenic substrate, methane gas bubbles together with colonies are often developed within an agar (Fig. 2b), which is also the sign of growth of methanogens.
9. PCR amplification, cloning, and sequencing of the 16S rRNA gene or the gene encoding methyl-coenzyme M reductase (*mcrA*) are also useful not only for detection but for phylogenetic characterization of methanogens, especially when making methanogenic enrichment culture. By doing so, one can know what types of methanogens are highly enriched.
10. Specific glassware for anaerobic cultivation is available from, for instance, Bellco Glass Inc. (http://www.bellcoglass.com/) and Sanshin Industrial Co. Ltd. (http://www.sanshinkogyo.co.jp/e/index.html).
11. If the culture tubes or vials do not resist pressure, to patch glass protection film on glassware is recommended.
12. If fast-growing methanogens are cultured, culture tubes should be vented manually.
13. Theoretically, one should sparge N_2/CO_2 in the dispensed medium and then add sodium bicarbonate in each tube. However, the way we describe here results in the same pH as that in the canonical way. Since the addition of sodium bicarbonate into each culture tube is time-consuming, we usually add it

before the medium solution is dispensed to each tube followed by N_2/CO_2 purging.

14. The addition of reducing agents and soluble methanogenic substrates in the latter steps results in the dilution of basal components. One can adjust the volume here to avoid the dilution.
15. Do this sterilization process every time when some kinds of solutions or gases are injected or removed through the butyl rubber.
16. Confirm that the medium becomes colorless (*see* also **Note 3**).
17. Prior to use, the dead space of the needle syringe is replaced with N_2 or N_2/CO_2 gas by pipetting a needle syringe with O_2-free gas from up close to gas outlet. The gas outlet is connected to the syringe filter (0.22-μm pore size) so that the O_2-free gas is sterilized by passing the filter.
18. If hydrogenotrophic methanogens are cultured using H_2/CO_2, the gas must be injected into the vial after inoculation and solidification are finished.
19. Be sure to use new needle syringe each time. Otherwise, a sample would not be diluted serially.
20. Gellan gum can be used as a solidifying agent instead of agar because of its high thermal stability. The use of gellan gum is described on Subheading 3.5. It is important to note that using gellan together with marine medium (containing high salt concentrations) is very difficult because gellan is immediately solidified right after mixing with marine medium. To isolate thermophilic methanogens from marine environments, the use of deep agar slant (*see* Subheading 3.4) or dilution-to-extinction method (*see* Subheading 3.2) is recommended.
21. We use Noble agar but other commercial agar can be also used.
22. Remove the colored component (soluble impurities) of the agar.
23. Inject and mix gently so as not to make gas bubbles in agar.
24. By following this procedure, the concentration of medium composition decreases by half. Accordingly, one can add two-fold concentration of methanogenic substrate in the initial preparation of the medium. To our knowledge, the decrease in the concentrations of the other medium compositions, such as N and P sources, does not seem to dramatically affect the growth of colony.
25. In our laboratory, sterilized and reduced liquid medium in a 20-mL test tube is used for the serial dilution of cell suspension.

26. Alternatively, the inoculum is mixed with the molten agar by pipetting using the needle syringe.
27. Keep it horizontal to make a homogeneous agar roll.
28. Pasteur pipettes plugged with cotton is desirable to prevent contamination.
29. Sometimes, the Pasteur pipette is connected to a gas line, and O_2-free gas is continuously flowed during all steps of colony manipulation to prevent oxygen exposure.
30. Do not touch the separated water present in the tube with the pipette tip.
31. Close O_2-free gas flow from a gas line before picking colony. Picked colony can also be ejected from the tip by pressing the gas flow.
32. Be careful not to touch nontargeted colonies during inserting needle to agar.
33. Mix well by pipetting to confirm the picking colony being into a fresh medium.
34. Gellan gum (Gelrite) is a water-soluble polysaccharide. Harris [23] reported that gellan gum is superior to agar as a solidifying agent for cultivation of some methanogenic species. Because of its thermal stability, solid culture using gellan gum is more suitable for thermophilic methanogens. However, roll tube method using gellan gum is difficult as gellan gum is quickly solidified during tube-rolling process.
35. The concentration of gellan gum in a medium is adjusted based on the cultivation temperature. Nakamura et al. used higher concentration of gellan gum when thermophiles are cultured [5].
36. The temperature for keeping a gellan gum in an unsolidified state depends on the temperature sensitivity of strain to be cultured. For culturing psychrophiles and mesophiles, the temperature is set to lower (45°C) to minimize heat stress.
37. To prevent exposure of oxygen, the operation under anaerobic chamber conditions is more desirable (but not essential).
38. Handling on ice is recommended to avoid heat stress for psychrophilic and mesophilic methanogens.
39. When cultivating hydrogenotrophic methanogens, H_2/CO_2 gas is used instead of N_2/CO_2.
40. *S. fumaroxidans* can be cultivated in pure culture when fumarate is present as a terminal electron acceptor (fumarate respiration).

5 Concluding Remarks

Except for several species, methanogens are still one of the most difficult microorganisms to cultivate and isolate. Recent cultivation-independent high-throughput massive sequencing studies reveal that a plethora of methanogenic species that remain uncultivated are widespread in any anoxic environments. Tremendous efforts have been made to isolate methanogens over the last decades, but the challenge still continues. The advent of new isolation techniques together with subsequent genomics, biochemistry, and ecological studies will unveil the true entity of methanogens.

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Cultivation of Methylotrophs

Donovan P. Kelly, Julie K. Ardley, and Ann P. Wood

Abstract

Methylotrophy is the ability of numerous *Alpha-*, *Beta-*, and *Gammaproteobacteria*, Gram-positive bacteria, Actinomycetes, and yeasts to obtain all the energy and carbon required for growth from the metabolism of methyl compounds which have no carbon–carbon bonds. Numerous media and techniques for the isolation and culture of methylotrophs have been described, and a comprehensive description of these protocols is given, with substrates such as methanol, methylamines, and methylated sulfur compounds, and the description of some more complex media. The metabolic and habitat diversity of methylotrophs is outlined, ranging from soil and freshwater organisms, through thermophiles and yeasts, to animal hosts and plant colonisers, including specialised types that induce (and reside in) root nodules in some plant species. The methods for the commercial exploitation of some methylotrophs are outlined. This survey excludes the methanotrophs, growing primarily on gaseous methane, as the cultivation of these is considered elsewhere.

Keywords: *Bacillus*, *Hyphomicrobium*, Methanol, Methylamines, Methylated sulfur compounds, *Methylobacterium*, Plants, *Proteobacteria*, Rhizobia, Yeast

1 Introduction

Methylotrophic organisms are defined as those capable of growth on one-carbon compounds containing methyl groups [1, 2]. The simplest hydrocarbon substrate is methane (CH_4), which is used by specialised types of organisms, the methanotrophs, most of the known isolates of which are incapable of growth on virtually any other substrate, except methanol [3–5]. This chapter is concerned exclusively with the cultivation of methylotrophs which do not grow on methane: the methanotrophs are dealt with elsewhere (Svetlana Dedysh, in this volume). Substrates commonly used by diverse methylotrophs are methanol (CH_3OH), methylamine (CH_3NH_2; MMA), dimethylamine [$(CH_3)_2NH$; DMA], trimethylamine [$(CH_3)_3N$, TMA], methanethiol (CH_3SH; MT), dimethylsulfide [$(CH_3)_2S$; DMS], dimethyldisulfide [$(CH_3)_2S_2$; DMDS], dimethylsulfoxide [$(CH_3)_2SO$; DMSO], dimethylsulfone [$(CH_3)_2SO_2$; $DMSO_2$], methanesulfonate (CH_3SO_3H; MSA), and *N*,*N*-dimethylformamide [$(CH_3)_2NC(O)H$; DMF]. 1,3,5-triazines can

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also be regarded as potential C_1 substrates: these are heterocyclic compounds with C–N–C–N–C–N ring structures, but have yet to be fully assessed as methylotrophic substrates. Most methylotrophs will use other non-methyl C_1 compounds such as formaldehyde (HCHO) and formic acid (HCOOH) for growth, and some use thiocyanate (SCN^-), carbon disulfide (CS_2), or carbonyl sulfide (COS) as a nitrogen or energy source [6, 7]. Virtually all organisms capable of methylotrophic growth on one or more of these compounds will also grow on standard heterotrophic media as chemo-organotrophs. Carbon for growth is assimilated from the C_1 substrates principally by one of two pathways in aerobic methylotrophs: the ribulose monophosphate (RuMP) cycle or the serine cycle. The latter also requires the assimilation of carbon dioxide, but such methylotrophs should not be classed as 'autotrophic' (i.e. able to grow with CO_2 as a sole source of carbon). Some methylotrophs are, however, able to oxidise methanol, MMA, and other C_1 compounds, such as formate, as sources only of energy, which is used to fix CO_2 by the autotrophic Calvin ribulose bisphosphate (RuBP) cycle, in a process called methylotrophic autotrophy [8–12].

The existence of non-methanotrophic methylotrophs seems first to have been reported in the nineteenth century [13]. Methylotrophs are seemingly ubiquitous, having been isolated from marine, freshwater, and terrestrial habitats ranging from plants, animals, peat bogs, tundra permafrost, hot spring waters, Antarctica, and the human body [2, 14–30]. Methylotrophy is not a clearly defined taxonomic criterion as once thought, but occurs widely across the range of Bacteria, and some Eukarya, and consequently has evolutionary and phylogenetic significance. The most well studied are the aerobic 'pink-pigmented facultative methylotrophs' (PPFMs; *Methylobacterium* species), *Paracoccus* species, facultatively anaerobic *Hyphomicrobium* species, some methylotrophic autotrophs, a small number of *Bacillus* species, and some yeasts.

The purpose of this chapter is to provide details of the methods and media used to isolate and culture a wide range of methylotrophs from diverse habitats.

2 Materials

Components of media are specified in the recipes given in the Sect 3. No specific proprietary sources are provided for most protocols, as materials such as yeast extract, peptone, and agar are available at similar quality from diverse suppliers. Although 'Difco' and 'Oxoid' are among the suppliers named in some recipes, this does not indicate that products from those suppliers are necessarily mandatory. Other inorganic and organic components should obviously be of the highest purity commercially available, but further purification is rarely required. A special case may be the need to wash commercial

agar to remove free sugars or other impurities that might support heterotrophic growth, when rigorous proof of growth only on an added methylotrophic substrate is being determined. The methylamines (MMA, DMA, and TMA) are normally added to media as their hydrochlorides. The use of distilled or deionised water is to be presumed for all media preparations.

3 Methods

3.1 Media Suitable for the Isolation and Culture of Most Methylotrophs

Targeted isolation of methylotrophs requires that the medium used to recover them from environmental samples contains only a C_1 compound as the source of carbon and energy to support growth. Typically a minimal mineral medium is supplemented with one of methanol, methylamine, dimethylsulfone, or whatever C_1 compound has been chosen for elective enrichment of organisms capable of growth on specific substrates. Many methylotrophs have, however, been isolated as heterotrophs using rich media, not necessarily containing a C_1 compound, and only subsequently shown to have methylotrophic growth potential. Heterotrophic and methylotrophic media in common use are described below.

3.2 Commonly Used Heterotrophic Media

Commercially available media on which methylotrophs have been isolated and maintained include tryptic soy broth (TSB) [31], peptone yeast extract glucose agar (PYG), R2A medium [32], and nutrient agar (and broth). In some cases media with a heterotrophic constituent (e.g. glycerol, sorbitol) have been supplemented with C_1 substrates. The preparation of some of these, and of glycerol–peptone (GP) medium, and '½ Lupin Agar' (1/2LA) is shown below.

This contains:

Yeast extract	0.5
Difco Proteose Peptone No. 3	0.5
Difco Casamino acids	0.5
D-Glucose	0.5
Soluble starch	0.5
K_2HPO_4	0.3
Sodium pyruvate	0.3
$MgSO_4.7H_2O$	0.05
Agar	15
Distilled water	1,000 ml

3.2.1 R2A Medium [32]

This will support growth of most methylotrophs and is a useful heterotrophic maintenance medium. It has been used for the isolation of some strains later shown to be methylotrophs. It can be used

at full strength (or double strength, as R3A) or in various dilutions (10% and 50%) and contains (g):

Final pH 7.2; this can be adjusted by addition of crystalline K_2HPO_4 or KH_2PO_4 before adding the agar. The original recipe recommends boiling to dissolve the agar before autoclaving at 121°C for 15 min.

3.2.2 Glycerol Peptone (GP) Agar

This can be used for maintaining stocks of pure cultures and contains (g l^{-1}):

Agar	15
Glycerol	10
Difco Peptone	10

Adjust to pH 7.0 with 1 M NaOH before making up to one litre and autoclaving.

3.2.3 Peptone Yeast Extract Glucose (PYG) Medium

Used for maintenance of species such as *Paracoccus* [33] and contains (g):

Peptone	5
Yeast extract	5
D-Glucose	5
Agar	10–15

Distilled water added to 1,000 ml after adjustment to pH 7.0 with 1 M NaOH.

3.2.4 ½ Lupin Agar (1/2LA)

This medium has been used especially for methylotrophs isolated from some plant root nodules, as detailed later [34, 35], and is best prepared as follows:

Dissolve these constituents (g) in 800 ml distilled water:

Mannitol	5.0
D-Glucose	5.0
Yeast extract	1.25
$MgSO_4.7H_2O$	0.8
NaCl	0.1
$CaCl_2.2H_2O$	0.2

Make separate stocks (g l^{-1}) of:

K_2HPO_4	0.87
KH_2PO_4	0.68
$FeSO_4.7H_2O$	0.5

Add 20 ml of each phosphate and 10 ml of the iron stock to the above solution.

Prepare a trace metal solution as below (g l^{-1}), and add 1 ml to the main medium:

$Na_2B_4O_7.10H_2O$	2.34
$MnSO_4.4H_2O$	2.03
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$Na_2MoO_4.2H_2O$	0.126

Adjust to pH 6.8 with 1 M NaOH, and add agar (12–15 g) if required, and make up to 1,000 ml with distilled water. Autoclave at 121°C for 20 min.

3.2.5 JMM Medium [36]

This defined medium can be prepared for use over a range of pH, using commercially available buffers at 20 mM: for pH 9.0, CHES (4.146 g l^{-1}); pH 7.0, HEPES (4.766 g l^{-1}); pH 5.5, MES (3.904 g l^{-1}); and pH <5.5, HOMOPIPES (6.328 g l^{-1}). The appropriate buffer is selected and prepared in 900 ml reverse osmosis water containing carbon and nitrogen substrates as below (g):

D-Galactose	1.8
L-Arabinose	1.5
L-Glutamate (disodium salt)	0.507

To this are added:

1 ml 1 M $CaCl_2.2H_2O$ (stock solution, 147 g l^{-1})

0.2 ml 0.1 M $FeCl_3.6H_2O$ in 1 M HCl (stock solution, 27 g l^{-1})

10 ml of this trace element solution (g l^{-1}):

$Na_2MoO_4.2H_2O$	0.096
Na_2SO_4	10.0
$MgSO_4.7H_2O$	24.64
$MnSO_4.4H_2O$	0.1114
$ZnSO_4.7H_2O$	0.108
$CuSO_4.5H_2O$	0.05

The mixture is adjusted to the desired pH with NaOH or HCl and made up to 990 ml with reverse osmosis water and autoclaved at 121°C for 20 min.

To the cooled medium are added 10 ml of filter-sterilised mixed phosphates (g l^{-1}: KH_2PO_4 2.04, K_2HPO_4 2.61) and 1 ml of a filter-sterilised vitamin solution (*see* **Note** 7), containing (mg ml^{-1}) biotin 0.02, thiamine 1.0, pantothenic acid 1.0).

Glutamate can be replaced as the nitrogen source by 10 mM NH_4Cl. For agar medium, the total volume before autoclaving can be reduced to 790 ml, and the agar (15 g) is separately autoclaved in 200 ml reverse osmosis water and mixed into the bulk medium.

3.2.6 Methanol Yeast Nitrogen Base (YNB) Broth

This medium is one of several suitable for the culture of methylotrophic yeasts [37–39], and contains 1% (v/v) methanol in 0.67% (w/v) Difco YNB without added amino acids (pH 5.4). Difco YNB contains (per litre):

$(NH_4)_2SO_4$	5 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g
NaCl	0.1 g
$CaCl_2.2H_2O$	0.1 g
HBO_3	500 μg
$CuSO_4.5H_2O$	40 μg
KI	100 μg
$FeCl_3$	200 μg
$MnSO_4.4H_2O$	400 μg
$Na_2MoO_4.2H_2O$	200 μg
$ZnSO_4.7H_2O$	400 μg
Biotin	2 μg
Calcium pantothenate	400 μg
Folic acid	2 μg
Inositol	2,000 μg
Niacin	400 μg
p-Aminobenzoic acid	200 μg
Pyridoxine hydrochloride	400 μg
Riboflavin	200 μg
Thiamine hydrochloride	400 μg

The pH of the medium can be modified by the addition of NaOH to the desired value.

3.2.7 Yeast Culture Medium of Choi and Kim [40]

This was used as the medium for cultures of *Pichia* for use in a bioreactor and contains (g l^{-1}):

Methanol	30
Glycerol	20
YNB	7 (or 15 for the bioreactor)
Yeast extract	20 (or 30 for the bioreactor)
Polypeptone	20 (or 30 for the bioreactor)
K_2HPO_4	5
$(NH_4)_2SO_4$	0.1
$MgSO_4.7H_2O$	0.1
$FeSO_4.5H_2O$	0.25
$MnSO_4.4H_2O$	0.2
Biotin	0.2 mg
Complete medium is pH 6.5	

3.3 Media with a C_1-Compound as the Only Substrate

These are examples of widely used media for methylotrophs, some of which were devised for the growth of methanotrophs, but are suitable for many non-methanotrophic methylotrophs [3, 41].

3.3.1 Ammonium Mineral Salts (AMS) Medium (ATCC Medium 784 AMS)

KH_2PO_4	0.54 g
K_2HPO_4	0.7 g
$MgSO_4.7H_2O$	1.0 g
$(NH_4)_2SO_4$	0.5 g
$CaCl_2.2H_2O$	0.02
$FeSO_4.7H_2O$	4 mg
$ZnSO_4.7H_2O$	100 μg
$MnCl_2.4H_2O$	30 μg
H_3BO_3	300 μg
$CoCl_2.6H_2O$	200 μg
$CuCl_2.2H_2O$	10 μg
$NiCl_2.6H_2O$	20 μg
$Na_2MoO_4.2H_2O$	60 μg

Dissolve in distilled water, adjust to pH 6.8, and make up to 1 litre; add agar (15 g) for solid medium; autoclave, and when cool aseptically add 0.5 g methanol (sterilsed by filtration through a 0.2 μm pore-size membrane filter; *see* **Note** 7).

3.3.2 Modified Nitrate Mineral Salts (NMS) Medium [16]

This is prepared from these stock solutions:

1. Dissolve in 1,000 ml distilled water (pH 6.8).

Na_2HPO_4	3.6 g
KH_2PO_4	1.4 g

2. Trace metal solution (mg l^{-1}):

Tetrasodium EDTA	100
$ZnSO_4.7H_2O$	7
$MnCl_2.4H_2O$	3
H_3BO_3	30
$CoCl_2.6H_2O$	20
$CuCl_2.2H_2O$	1
$NiCl_2.6H_2O$	2
$Na_2MoO_4.2H_2O$	3

3. 0.05 g $FeSO_4.7H_2O$ dissolved in 100 ml 1 mM H_2SO_4. (Alternatively a commercial sequestered-iron complex, providing the same amount of iron, can be used).
4. 2% (w/v) $MgSO_4.7H_2O$
5. 0.2% (w/v) $CaCl_2.2H_2O$

NMS medium is prepared by dissolving 1 g KNO_3 in 840 ml distilled water, and adding 10 ml each of solutions 2–5, adjusting to pH 6.8, and autoclaving. After cooling to about 60°C, 20 ml of autoclaved solution 1 is added. To this complete minimal medium is aseptically added 100 ml sterilised substrate solution at 10 times the concentration required (e.g. 100 ml 150 mM MSA, 250 mM methanol, 200 mM MMA, or 100 mM dimethylsulfone: the methanol should be filter-stesilised (**Note** 7), but the other substrates are stable for autoclaving).

For solid media, NMS medium is gelled with high purity 1.5% (w/v) agar or a gelling agent of choice.

3.3.3 Marine Ammonium Mineral Salts (MAMS) Medium

This has been used for the isolation of methylotrophs from marine sources, but other media described in this chapter could also be employed, with the addition of NaCl at 20–30 g l^{-1}. It contains (g l^{-1}):

$(NH_4)_2SO_4$	1.0
$MgSO_4.7H_2O$	1.0
$CaCl_2.2H_2O$	0.2

(continued)

NaCl	20 or 30
FeEDTA	0.005
$Na_2MoO_4.2H_2O$	0.002
KH_2PO_4	0.36
Na_2HPO_4	1.91
Trace element solution*	1 ml l^{-1}
Vitamin solution**	1 ml l^{-1}

Autoclave the phosphates, filter-sterilise trace metals and vitamins, and add to the cooled sterile mineral salts medium; substrates are supplied as for AMS and NMS media and made up to a final volume of one litre.

Note that some methylotrophs, e.g. many *Methylobacterium* species, do not tolerate NaCl above 1.0–1.5% (w/v).

* Suitable trace element solutions can be any of those widely described in the literature (e.g. as in medium 3.3.6 described below [42]).

** A suitable general purpose vitamin mixture [42] for marine and non-marine bacteria is prepared by dissolving the following vitamins in about 500 ml distilled water (mg): thiamine-HCl, *p*-aminobenzoic acid (10 of each), nicotinic acid, pyridoxine-HCl, riboflavin, calcium pantothenate (20 of each), cyanocobalamin (0.5–1.0), and biotin [1]. The last three components should be dissolved in separate amounts of water and then mixed with the bulk solution. Adjust to pH 4.0 by careful addition of 0.1 M NaOH and then make up to one litre with water. This vitamin mixture can be stored frozen (unsterile) or refrigerated after filter-sterilisation (**Note 7**).

A variant of this medium (Medium 1313) is described in the list of media recommended by the DSMZ, including trace metal and vitamin mixture recipes (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1313.pdf).

3.3.4 Methanol Medium 72 (Belgian Coordinated Collections of Microorganisms; http://bccm.belspo.be/index.php)

This has been used for the isolation and culture of rhizobial methylotroph symbionts from some leguminous plants and contains per litre of distilled water (final pH 7.0):

K_2HPO_4	1.2 g
KH_2PO_4	0.62 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g

(continued)

$CaCl_2.2H_2O$	34 mg
$FeCl_3.6H_2O$	1 mg
Trace element solution	1 ml
Agar	15 g

The trace element solution contains (mg l^{-1}):

$CuSO_4.5H_2O$	5
$CoCl_2.6H_2O$	5
$MnSO_4.H_2O$	7
$Na_2MoO_4.2H_2O$	10
H_3BO_3	10
$ZnSO_4.7H_2O$	70

After sterilisation, cool to 45°C and aseptically add 10 ml filter-sterilised methanol (**Note** 7).

3.3.5 Colby and Zatman Trimethylamine Medium 96 (Belgian Coordinated Collections of Microorganisms)

This is prepared as for Methanol Medium 72 (above), but replacing the methanol with 10 ml filter-sterilised 10% (w/v) trimethylamine hydrochloride, and is suitable for some trimethylamine users [43].

3.3.6 Paracoccus versutus Medium (PVM; pH 7.3–8.4) [42]

This medium has been repeatedly and successfully used for isolation and maintenance of methylotrophs including several species of *Paracoccus*, *Hyphomicrobium*, *Arthrobacter*, *Xanthobacter*, *Methylobacterium*, *Bacillus*, *Brevibacterium*, *Afipia*, and *Micrococcus* [11, 17, 18, 20, 21, 44, 45]. It has a high concentration of phosphate buffer, making it particularly suitable for use with substrates where a change in pH occurs during metabolism (e.g. DMS, MSA, and formate), but would be unsuitable for any strains inhibited by high phosphate concentrations. It is prepared from stock solutions, which can be stored indefinitely, as below:

1.

$Na_2HPO_4.2H_2O$	39.5 g
KH_2PO_4	7.5 g

Dissolve, with stirring, in distilled water to make one litre. (Alternative disodium hydrogen phosphates are the dodecahydrate, 79.5 g, or the anhydrous salt, 31.5 g.)

2. 4% (w/v) NH_4Cl
3. 4% (w/v) $MgSO_4.7H_2O$

4. Trace metal solution [42]: prepared in a 1–2 litre beaker on a magnetic stirrer with 400 ml water, 50 g ethylenediaminetetraacetic acid (EDTA, disodium salt), and 9 g NaOH. Dissolve the following metal salts (g) individually in 30–40 ml aliquots of water and add to the EDTA solution (with 5-10 ml washings):

$ZnSO_4.7H_2O$	5
$CaCl_2$	5 (or 7.34 g $CaCl_2.2H_2O$)
$MnCl_2.6H_2O$	2.5
$CoCl_2.6H_2O$	0.5
Ammonium molybdate	0.5
$FeSO_4.7H_2O$	5.0
$CuSO_4.5H_2O$	0.2

Adjust to pH 6.0 by the gradual addition of 1 M NaOH (ca. 24 ml) with stirring. Make up to one litre with distilled water. This should be stored in a dark bottle at room temperature; the solution is initially pale green but turns brown with time: this does not affect its use. The stock solution should not be heat-sterilised unless diluted in culture media, but it can be filter-sterilised and added to sterile culture media.

For preparation of liquid cultures, to avoid precipitation of phosphates and to prevent decomposition of growth substrates, this medium has to be made in three parts:

Part I: solution 2, 10 ml; solution 3, 2.5 ml; trace metal solution 4, 10 ml; distilled water, 550 ml.

Part II: solution 1, 200 ml (with or without NaOH, *see* below).

Part III: this is the growth substrate in 200 ml water, at five times the final concentration needed in the culture medium. After autoclaving separately at 110°C for 10 min and cooling, the three parts are mixed aseptically prior to use. The final mixture is pH 7.3, but can be modified by adding 1 M NaOH to Part II: 10 ml gives about pH 8.4 and pH can be adjusted by different amounts of NaOH added to Part II or of NaOH or Na_2CO_3 added to the complete medium.

Agar medium for maintenance slopes or plates is prepared with solution 1 (200 ml), solution 2 (10 ml), solution 3 (2.5 ml), solution 4 (10 ml), and agar (15 g) to give medium at pH 7.3: for medium at pH 8.4, add 11.5 ml 1 M NaOH. Add substrate solution, make up to one litre with distilled water, add 15 g agar, and autoclave. Heat-stable C_1 substrates may be added at the appropriate concentration prior to autoclaving. Volatile or heat-sensitive substrates should be filter-sterilised (*see* **Note** 7) then added to the sterilised agar mixture and swirled to mix thoroughly before pouring plates or slopes.

Where culture pH may change during growth on the substrate, addition of 10 ml saturated phenol red solution to the agar medium will indicate pH change (pink to orange to yellow for acid or to very dark pink for alkali production).

3.3.7 Methanol Medium for Hyphomicrobium [16]

This contains (g l^{-1}):

KH_2PO_4	1.35
Na_2HPO_4	2.13
$(NH_4)_2SO_4$	0.5
KNO_3	5.0
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.005
$FeSO_4.7H_2O$	0.002
$MnSO_4.7H_2O$	0.0005

Dissolve in one litre distilled water, autoclave, and when cool add 5 ml methanol and 21 ml of filter-sterilised vitamin solution containing (mg 100 ml^{-1}) biotin, calcium pantothenate (10 each), *p*-aminobenzoic acid [20], thiamine dichloride [30], niacin [35], and vitamin B_{12} [5] (*see* **Note 7**). This medium is suitable for enrichment and culture of both aerobic and anaerobic *Hyphomicrobium* strains.

3.3.8 Mineral Medium for Enrichment of Thermotolerant Bacillus Species [46]

This medium was used for the isolation of methylotrophic *Bacillus* strains using continuous flow chemostat culture at 50°C and pH 6.8 and contains (per litre):

K_2HPO_4	3.8 g
$Na_2HPO_4.H_2O$	2.8 g
$(NH_4)_2SO_4$	3.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.2H_2O$	5.3 mg
$MnSO_4.H_2O$	200 μg
$ZnSO_4.7H_2O$	200 μg
$Na_2MoO_4.2H_2O$	47 μg
$CoCl_2.6H_2O$	40 μg
$CuSO_4.5H_2O$	40 μg
H_3BO_3	30 μg
Methanol	5.0 g (added aseptically after sterilisation)

3.3.9 Mineral and Vitamin (MV) Medium for Gram-Positive Methylotrophs [16]

This is prepared from four stock solutions containing:

1. Make up to one litre after adjustment to pH 7.0 with HCl.

KH_2PO_4	38 g
NaH_2PO_4	28 g
$(NH_4)_2SO_4$	36 g

2. Trace metals solution (autoclaved separately) containing (g l^{-1} in 1 mM H_2SO_4):

$FeCl_3$	2.0
$CaCl_2.2H_2O$	5.3
$MnSO_4.H_2O$	0.2
H_3BO_3	0.03
$CuSO_4.5H_2O$	0.040
$ZnSO_4.7H_2O$	0.2
$Na_2MoO_4.2H_2O$	0.047
$CoCl_2.6H_2O$	0.04

3. Magnesium sulfate solution (autoclave separately):

$MgSO_4.7H_2O$	246.5 g l^{-1}

4. Vitamin solution (mg l^{-1}; filter-sterilise and store in the dark at 4°C; **Note** 7):

Biotin	200
Vitamin B_{12}	10

The medium consists of 800 ml distilled water plus 100 ml solution 1, which is autoclaved and cooled. After cooling, sterile trace metals (1 ml), magnesium sulfate (1 ml), and vitamin solution (0.1 ml) are added. Substrate (e.g. methanol) is added as 100 ml of a solution of appropriate concentration. Add 1.5% (w/v) agar for solid medium.

3.3.10 Ammonium Nitrate Mineral Salts (ANMS) Minimal Medium

This medium has been employed for enrichment culture of methanotrophs and can be used for the culture of non-methanotrophic methylotrophs if supplemented with methanol or other C_1 substrates. It is not a medium recommended by the authors for routine use, as the inclusion of both nitrate and ammonium as nitrogen

sources is a potential disadvantage as they are unlikely to be consumed simultaneously, and biphasic growth of cultures could occur if the use of nitrate required expression of nitrate reductase after exhaustion of the ammonium. It contains (g l^{-1}) KNO_3, 0·25; NH_4Cl, 0·25; KH_2PO_4, 0·13; $Na_2HPO_4.12H_2O$, 0·358; $MgSO_4.7H_2O$, 0·4; $CaCl_2$, 0·1; ferric-EDTA, 0·00019; and $Na_2MoO_4.6H_2O$, 0·00013. To this mixture are added 0·5 ml of a stock solution of trace elements, containing (g l^{-1}): $CuSO_4.5H_2O$, 0·2; $FeSO_4.7H_2O$, 0·5; $ZnSO_4.7H_2O$, 0·4; H_3BO_3, 0·015; $CoCl_2.6H_2O$, 0·05; disodium EDTA, 0·25; $MnCl_2.4H_2O$, 0·02; and $NiCl_2.6H_2O$, 0·01. The pH of the medium is adjusted to pH 6·8 with NaOH. Optionally, 2·5 ml of a filter-sterilised vitamin stock solution can be added, containing (mg l^{-1}) thiamine hydrochloride, 10; nicotinic acid, 20; pyridoxamine, 10; *p*-aminobenzoic acid, 10; riboflavin, 20; biotin, 1; and cyanocobalamin (vitamin B_{12}), 1 (*see* **Note 7**).

3.4 Basic Enrichment and Isolation Techniques for Methylotrophs (see Notes 1–8)

As for most bacteria, these can include shaken and static flask cultures, continuous flow chemostats, and filled or partially filled bottles or tubes as appropriate for aerobes, anaerobes, and microaerophiles and selection on media solidified with agar. Anaerobic enrichments require an alternative electron acceptor, and nitrate is used to obtain denitrifying methylotrophs capable of anaerobic growth, for example, of *Paracoccus* or *Hyphomicrobium*, on methanol or formate. Chemostat cultures should be gassed with air, with or without added CO_2 or N_2/CO_2 as appropriate for the enrichment. Enrichment cultures are usually successful within days or a few weeks depending on the inoculum size and growth rate but can take several weeks or even months in the case of slow-growing organisms either because of their metabolism, low temperatures, or suboptimal conditions being used. Some environmental samples will inevitably contain more microorganisms than others: a gram of soil will produce methylotrophs (and probably any other metabolic types) more quickly than a swab taken of the mouth or skin, for example, where the sample size will usually be much smaller.

The diversity of substrates used by different methylotrophs means that the choice of enrichment substrate may depend on the chemistry of the environment being sampled, if there is a particular C_1 compound of interest prevalent there, or substrates may be chosen to recover organisms with particular metabolic properties. The concentration used for any substrate will be determined in part by its toxicity (if any) and potential to change pH during its metabolism. Typically substrates tend to be used at concentrations (mM) of 1–2 mM DMS, dimethyldisulfide, or MT, 10 mM dimethylsulfone, 10–15 mM MSA, 10–50 mM methanol, 10–20 mM MMA, 5–10 mM DMA or TMA, and 25–50 mM

formate (**Note** 7). The methylamines (MMA, DMA, TMA) are normally supplied as their hydrochlorides. Formaldehyde (HCHO; 1 mM) should be provided as the ultrapure methanol-free compound, which can be prepared from paraformaldehyde, or purchased as a 16% (w/v) ultrapure EM grade solution in sealed 10 ml ampoules (www.antibodies-online.com). Dimethylsulfoxide seems to be tolerated and optimally supplied at about 10–20 mM, but this must be as the ultrapure chemical, free of impurities such as toxic DMS. Gaseous halomethanes such as chloromethane are typically supplied at 2–5% (v/v) in air, both in sealed vessel cultures and as flowing gases for fermenter cultures.

Most substrates can be autoclaved, but volatile and/or toxic compounds are normally filter-sterilised (**Note** 7) and added to the basal media (or agar media) separately or on filter paper (**Note** 7).

3.5 Survey of the Varieties of Methylotrophs, Their Habitats, and Additional Procedures for Their Isolation and Study

These organism types can be broadly defined by their physiological properties and typical habitats, as summarised in the following sections. Procedures for the special case of rhizobial methylotrophs in plant root nodules are considered in detail.

3.5.1 Methylotrophic Aerobic Proteobacteria Found in Soil and Water

These include species of *Methylobacterium*, *Paracoccus*, *Pseudomonas*, *Hyphomicrobium*, *Sphingomonas*, and *Klebsiella*, as detailed below. The most environmentally diverse and exhaustively studied of the non-methanotrophic methylotrophs are *Methylobacterium* species, which comprise the 'aerobic Gram-negative pink-pigmented facultative methylotrophs' (PPFMs). At the time of writing, at least 49 species names had been validated [47–49], and their diversity and properties have been comprehensively reviewed [51]. They are facultatively heterotrophic and will grow on methanol and variously on a range of other C_1 compounds, including MMA, using the serine pathway [50]. *Methylobacterium* species are ubiquitous in nature, being found in soil and freshwaters, and as residents of plants and animals.

Several facultatively methylotrophic species of the genera *Paracoccus*, *Hyphomicrobium*, *Xanthobacter*, *Methylopila*, *Methylophaga*, *Methylophilus*, *Methylobacillus*, *Methylovorus*, *Methylorhabdus*, *Ancyclobacter* (*Microcyclus*), *Klebsiella*, *Acetobacter*, *Methyloversatilis*, *Methylibium*, *Methylotenera*, and *Beijerinckia* have been shown to use methanol or MMA, and in many cases one or more of dimethylsulfone, DMSO, DMS, formaldehyde, and formate for growth [2, 20, 24, 44, 51–54]. Some species of *Aminobacter*, *Methylobacterium,* and *Leisingeria* are able to grow methylotrophically on halomethanes and MMA [55, 56]. Two novel genera from soil and marine sources were isolated using methanesulfonate (MSA) as a

methylotrophic substrate, *Methylosulfonomonas* and *Marinosulfonomonas*, which are also methylotrophic on other C_1 compounds. MSA has become recognised as a substrate for numerous environmental isolates [14, 21, 57–59] (M Cunliffe and AP Wood, unpublished data).

Notably some strains of *Hyphomicrobium*, *Methylobacillus*, *Methylophaga*, and *Methylovorus* are obligately methylotrophic and may show a restricted obligately methylotrophic substrate range and are able to grow only on methanol or MMA [2, 60, 61]. Other species of *Hyphomicrobium* are metabolically very versatile, growing on MSA, DMS, DMSO, dimethylsulfone, and other C_1 compounds [45, 60]. Other metabolically versatile species include *Starkeya novella*, *Paracoccus versutus*, *P. pantotrophus*, and some strains of *P. denitrificans* and of *Xanthobacter*, which grow on numerous heterotrophic substrates, and in some cases as chemolithoautotrophs on thiosulfate or hydrogen, and on unusual substrates such as substituted thiophenes [51, 62, 63]. *Paracoccus*, *Xanthobacter*, *Starkeya*, and *Beijerinckia* are all methylotrophic autotrophs that use the Calvin RuBP cycle for CO_2 fixation, whereas *Methylophilus*, *Methylobacillus,* and *Acetobacter* use the RuMP cycle and *Hyphomicrobium*, *Methylosulfonomonas*, *Marinosulfonomonas*, and probably *Klebsiella* use the serine pathway for C_1 assimilation. While many of the methylotrophs described above (including *Methylobacterium*) are *Alphaproteobacteria*, species of some genera of *Betaproteobacteria* and *Gammaproteobacteria* have methylotrophic potential: for example, *Methylibium* and *Methyloversatilis* which are members of the betaproteobacterial order *Burkholderiales* and the betaproteobacterium *Methylophaga*, which uses the RuMP pathway and can grow on DMS as well as methanol [23, 53, 54, 64, 65]. *Burkholderiales* expresses a methanol dehydrogenase, encoded by the *mdh2* gene, which differs from the *mxaF* gene (and encoded protein) of the *Alphaproteobacteria* [54]. Most unexpected among these methylotrophs is the gammaproteobacterium *Beggiatoa*, which is a multicellular, filamentous, gliding bacterium, some species of which are obligate autotrophs, found in sulfide-gradient habitats, but which can grow on methanol by an as yet undetermined mechanism [66]. The actinomycete *Amycolatopsis* is also methylotrophic [67]. A novel 'mixotrophic' combination of methylotrophy and chemolithotrophy is exhibited by some strains of the betaproteobacterium *Thiobacillus thioparus*, which can grow on DMDS, DMS, or MT, obtaining energy from the oxidation of both the methyl and sulfide groups of these compounds to drive autotrophic growth using the RuBP cycle [68].

Methylotrophy has also been shown in strains of *Afipia felis*, some of which can use MSA, dimethylsulfone, MMA, and DMA for growth; in *Flavobacterium* from the Antarctic [20, 21]; in *Variovorax* from the human mouth [18]; and in *Klebsiella*, *Achromobacter*, *Ralstonia,* and *Pseudomonas* strains from several sources [24, 29, 30].

The plant-pathogenic alphaproteobacterium *Sphingomonas melonis* was also shown to be methylotrophic on methanol, but not other methyl compounds [24], and stable isotope probing has also implicated *Sphingomonas* strains in methylotrophy in natural habitats [69].

A growing diversity of marine methylotrophs is being discovered, such as strains of *Methylophaga* and several novel clades of *Gammaproteobacteria* that grow on methanol, MMA, DMA, and DMS, as well as marine populations with strains of uncharacterised taxa, *Alphaproteobacteria*, and the *Cytophaga* (CFB) group, which assimilate methyl bromide and other C_1 substrates [23, 70]. The phytoplankton osmolyte dimethylsulfoniopropionate [DMSP; $(CH_3)_2S^+CH_2CH_2COO^-$] is the principal source of marine DMS (from the microbial scission of DMSP to DMS and acrylate), on which numerous marine bacteria grow [23, 70]. DMSP can itself also be a substrate for methylotrophs: an early DMSP isolate was a bacterial strain from an intertidal mud, which quantitatively demethylated DMSP and 3-methiolpropionate to 3-mercaptopropionate, and grew on both DMSP and glycinebetaine, as well as on their methylated degradation products: dimethylglycine, sarcosine, methylamines, and DMS [71].

While typically aerobic, species of *Paracoccus* and *Hyphomicrobium* are facultative denitrifiers, able to grow on formate or methanol using nitrate or nitrous oxide as terminal electron acceptors, and can be enriched and isolated in this way from environmental samples (**Note 6**).

3.5.2 Isolation of Methylotrophs from Plants Leaves and Stems (the Phyllosphere)

These techniques have been employed primarily for the detection, isolation, and culture of species of *Methylobacterium* (PPFMs), which are found in abundance on plant leaf and stem surfaces, and on which there is an extensive literature [49, 72–74]. Numerous species and strains of *Methylobacterium* have been isolated from plants [49, 75]. A standard procedure for the recovery of PPFMs is to make leaf impressions by pressing leaves on to the surface of plates of NMS–methanol agar medium supplemented with cycloheximide at 100 mg l^{-1} (to inhibit fungal growth) for 1–10 min before removing the leaves. Pink and other colour colonies will appear within 1 week and can be purified by standard procedures. Bacteria both on and in the leaf (or stem samples) can be recovered by washing the sample with sterile water then grinding the material with glass beads in sterile water and plating dilutions on to the same medium. To recover organisms with greater physiological diversity, elective conditions can be varied by changing the methanol concentration (0.05–0.2% v/v), the pH (pH 4–9), or the temperature (4–50°C) and by supplementing the medium with a vitamin solution.

3.5.3 Isolation and Cultivation of Methylotrophs from Plant Root Nodules, Infection of Roots with Nodule-Inducing Methylotrophs, and Re-isolation of the Organism from Induced Nodules

Methylotrophic rhizobia were first described in 2001 by Sy et al. [76], who demonstrated that non-pigmented strains isolated from several species of *Crotalaria* in Senegal were able to use methanol as a sole carbon source: methylotrophy contributed to the effectiveness of the symbiosis with *Crotalaria podocarpa* [77]. Phylogenetic analysis of the 16S rRNA gene indicated that these strains were most closely related to species of *Methylobacterium*, with the novel group of rhizobia being named as *Mtb. nodulans* [78]. Subsequently, pink-pigmented rhizobia, isolated from the crotalarioid legume *Listia* (previously *Lotononis*) *bainesii* [79, 80], were also characterised as belonging to the genus *Methylobacterium* [81]. These strains are the specific microsymbionts of several species of *Listia* [35, 82]. Unlike the methylotrophic *Mtb. nodulans*, they are unable to use methanol or formaldehyde as sole carbon sources and lack the *mxa* operon common to other *Methylobacterium* species, which encodes enzymes for the primary oxidation of methanol [83].

Although the majority of described rhizobial genera (e.g. *Bradyrhizobium*, *Burkholderia*, *Ensifer*, *Mesorhizobium*, and *Rhizobium*) are able to fix nitrogen with diverse legume hosts [84], rhizobial methylobacteria appear to form symbioses exclusively with African crotalarioid legumes. Species within the tribe *Crotalarieae* vary in their symbiotic specificity: previous studies found that the plants nodulate with a range of taxonomically diverse rhizobia [85–87], while those species that form rhizobial symbioses with *Methylobacterium* are highly specific [35, 76, 82]. Although methylobacteria are uncommon legume symbionts, their symbiosis with the host plant appears to be mechanistically similar to that observed in other legume symbioses [88]. The genomes of *Mtb. nodulans* strain ORS 2060^T and the methylobacterial *Listia* symbiont strains 4–46 and WSM2598 [89, 90] all possess copies of the genes required for the synthesis of Nod factors, which are the signal molecules initiating most rhizobia–legume symbioses [91]. The Nod factors of ORS 2060^T are induced by several legume flavonoids [92]. The methodologies for isolating, growing, testing, and using rhizobial methylobacteria as inoculants on legumes are generally the same as those used for other rhizobial species. The first detailed compilation of these methodologies was given by Vincent [93] and updated by Somasegaran and Hoben [94]. Since 1985, advances in molecular and genetic techniques have led to the identification of many novel rhizobial species, and a new manual 'Working with Rhizobia' has been developed to provide practical knowledge of current methodologies for rhizobial technology [95]. The following summary, with emphasis on methylobacterial rhizobia, is based on this handbook.

Collection of Nodules

As with other species of root nodule bacteria, the isolation of rhizobial methylobacteria begins with the collection of nodules, either directly from legumes in the field or from trap hosts grown in the glasshouse on rhizosphere soil collected on-site. The

sampling location should be recorded with a reference map or a GPS device. As the type of rhizobia present in a soil can be greatly influenced by the clay content, pH, conductivity, and cation exchange saturation, it is recommended that the soil (100 g) is analysed for these properties. Nodules are best collected when the soil is moist, and the target legume must be identified at least to the genus level; a sample (preferably in flower) should be pressed for future identification of the species. Healthy plants should be selected and must be carefully excavated to avoid dislodging the nodules. Care must also be taken that exposed roots and nodules originate from the target legume; in perennial legumes the nodules are mostly found not on the woody main root but on fine surface roots or lateral roots. Isolation of rhizobia is usually more successful from functional (i.e. nitrogen-fixing) nodules; these are firm and when dissected are red or pink inside, due to the presence of leghaemoglobin. Nodules are best excised with a small amount of the root attached (2–5 mm), for ease of handling.

Nodules collected from remote areas can be stored and preserved in tubes containing desiccant material, such as silica gel, for transport to the laboratory. The desiccant acts to remove water from the nodules rapidly, and to keep them dry, preventing the growth of other microorganisms. A cotton plug separates the nodules from the desiccant in the bottom of the tube: nodules can be kept in this way for a maximum of 3 months.

Isolating Rhizobia from Nodules

Desiccated nodules are re-hydrated in water for 3–4 h. Re-hydrated (or fresh) nodules are surface-sterilised in 70% (v/v) ethanol for 1–2 min, followed by about 3 min in a 3–4% (v/v) solution of commercial sodium hypochlorite, to which Tween 80 (10 µl l^{-1}) can be added as a wetting agent: for very small nodules, these times may need to be reduced. Nodules are then rinsed in six changes of sterile deionised water. To release the rhizobia, each nodule is aseptically crushed (using a sterile glass rod, forceps, or a wooden applicator stick) in a drop of sterile water on the lid of a sterile Petri dish or in a microtitre plate. To improve the chances of obtaining rhizobia, rather than endophytic bacteria, the nodule should be crushed by pressing it once only. A loopful of the nodule exudate is aseptically streaked on plates of an appropriate growth medium, using a dilution streaking pattern to isolate single colonies. Media for isolations should be solid, undefined, and able to support the growth of a broad range of nodule bacteria. Examples include yeast mannitol agar (YMA) [93], tryptone yeast extract (TY) [96], and ½ Lupin Agar (described above): ½ LA allows differential colonial morphology of the diverse rhizobial species to be expressed. Plates are incubated at 28°C and checked every 24 h to observe growth; single colonies of the putative rhizobial isolates are then selected for subculture and purification.

Culture Conditions

Rhizobial methylobacteria grow readily as plate or broth cultures on all the recommended media, with best growth on succinate (20 mM), rather than a carbohydrate, as the carbon substrate. Rhizobial methylobacteria also grow well on peptone-based media such as nutrient agar; in contrast, many other species of rhizobia typically do not grow well on nutrient agar [93]. To determine specific nutrient requirements, or metabolic behaviour and growth products, a completely defined medium such as JMM [36] or Methanol Medium 72 is required, as described above.

As most species of *Methylobacterium* grow readily on organic acids but can only grow on a narrow range of carbohydrates [97], methylobacterial rhizobia are grown on JMM supplemented with sodium succinate (20 mM) in place of D-galactose or L-arabinose. Both JMM and Methanol Medium 72, with methanol as sole carbon source, support the growth of the methylotrophic *Mtb. nodulans* rhizobia [76, 83].

Authenticating and Assessing Nitrogen Fixation by Rhizobial Isolates

Surface-sterilised nodules may contain bacterial endophytes as well as the nitrogen-fixing symbiont [98], so pure nodule isolates must be shown to be able to nodulate the host legume, according to Koch's postulates. The development of elite rhizobial strains for commercial application in agriculture also requires an assessment of their ability to fix nitrogen optimally with the host legume species. The legume inoculant industry in Australia has developed strains of rhizobial methylobacteria for use with the pasture legume *Listia bainesii* [99]. Authentication and N_2 fixation experiments can be conducted using a variety of growth systems, for example, pots or enclosed vials filled with a sterile vermiculite or sand medium. The main requirements are:

1. No plant-available nitrogen is in the system except that contained in the seed.
2. The system is free of rhizobia other than those applied as inocula; i.e. hygiene must be maintained throughout the duration of the experiment.
3. A non-inoculated control and a control supplying chemically bound nitrogen in a non-limiting amount must be included.
4. Other nutrients required for plant growth are non-limiting.
5. The growth substrate is non-limiting for plant growth and allows nodulation.

Many legumes require pretreatment of the seeds to break dormancy before they will germinate. Examples of such treatments include scarification, or immersion in boiling water, or in

concentrated sulfuric acid [76] for various periods of time. Following pretreatment, the seeds are surface-sterilised by immersion in 70% (v/v) ethanol for 1–2 min, then 3 min in 3–4% (v/v) commercial sodium hypochlorite, followed by six rinses in fresh changes of sterile deionised water. Seeds are then germinated in the dark on 1% (w/v) aqueous agar plates, or between sterile moistened filter paper discs in sterilised plastic containers, at 15–20°C. Following germination, when the radicles are 0.5–1 cm long, the seeds are sown into pots or vials inside a laminar flow cabinet. Using a sterile rod, or applicator stick, holes are made around the perimeter of the container. Up to eight seeds per container may be sown and then covered and watered in to ensure close soil-radicle contact. Seedlings are later thinned, usually to four per pot, to ensure uniformity and optimal growth. Plants are inoculated as follows:

1. Add 0.5–1.0 ml sterile 1% (w/v) sucrose solution or deionised water to an agar plate culture of the rhizobia, and scrape the colonies off with a flamed spatula to produce a suspension.
2. Transfer the suspension to a sealable bottle containing sterile 1% (w/v) sucrose or deionised water, seal, and shake vigorously, or vortex, to ensure uniformity; the inoculant can also be supplied as liquid broth culture. Suspensions should contain 10^6 to 10^8 viable cells per ml, which is sufficient for nodulation.
3. With a sterile syringe or pipette tip, place a total of 0.5–1.0 ml of suspension directly on to the base of all the seedlings in each pot.
4. Disinfect the laminar flow cabinet and change the syringe or pipette tip between inoculation with each rhizobial strain.
5. After completion of the strain treatments, add 0.5 ml of sterile 1% (w/v) sucrose or sterile deionised water to the seedlings of the uninoculated and N-fertilised controls. Plants are harvested 4–10 weeks after inoculation and assessed for nodulation and, if determining N_2 fixation, above-ground biomass. Rhizobial strains are deemed to be fully effective for N_2 fixation if the shoot dry weight exceeds 75% of the N-fed controls. Authentication is confirmed if isolates nodulate the roots of inoculated plants and uninoculated plants remain nodule free; the identity of rhizobial strains re-isolated from the nodules can be confirmed by rep-PCR [100] or partial sequencing of the 16S rRNA gene.

Molecular Techniques Specific to Rhizobia

Full molecular characterisation of rhizobial species requires sequencing and phylogenetic analyses of their genes for nodulation

and nitrogen fixation: the key symbiotic loci. These genes are found only in rhizobial *Methylobacterium* strains and not in any other *Methylobacterium* species [76, 78]. Phylogenetic analysis of the *nodA* gene shows that while *nodA* of the rhizobial methylobacteria is most closely related to the *nodA* of diverse rhizobial species isolated from African legumes, the *nodA* sequence of the *Listia bainesii* methylobacterial strains is highly divergent relative to other *nodA* sequences [82].

3.5.4 Methylotrophic Gram-Positive Bacteria

Methylotrophy is being found in a growing number of Gram-positive bacterial genera: all of those studied to date are RuMP cycle methylotrophs [2, 18, 46, 101–103]. Methylotrophic species of *Arthrobacter*, *Bacillus*, *Nocardia*, *Corynebacterium*, and *Mycobacterium* have been known since the 1970s/1980s [33, 43, 101, 104–110]. An obligately aerobic mesophilic *Bacillus*, *B. methylotrophicus*, was isolated from rice rhizosphere soil using AMS medium supplemented with 0.5% (v/v) methanol and cycloheximide at 10 μg ml^{-1} [111]. This grew over the range 15–45°C and optimally at 28°C. Most of the methylotrophic *Bacillus* strains isolated to date have been thermotolerant or thermophilic, including *B. methanolicus* and *B. brevis*, which exhibit optimum growth on methanol in the range of 50–57°C [103, 109, 112]. *B. methanolicus* is a restricted methylotroph (growing only on mannitol as a sugar substrate), and in which, unusually, the genes for its methanol dehydrogenase and some enzymes of the RuMP cycle are plasmid-borne [113]. The methylotrophic bacilli do not use the PQQ-methanol dehydrogenase [EC 1.1.99.8] common to the Gram-negative *Proteobacteria*, but contain an NAD-dependent methanol dehydrogenase (EC 1.1.1.244) [46] and have genes encoding four enzymes of the tetrahydromethanopterin C_1-oxidation pathway [2, 66]. Enzymes for methanol oxidation in the bacilli and *Burkholderiales* thus seem to have arisen by convergent evolution, rather than from a common methylotrophic ancestor [54].

B. methanolicus has been used for the potential commercial production of lysine and glutamic acid from methanol at 50°C [114, 115], the latter being the subject of an international patent (WO/1999/020785).

Nazina [116] reported a methylotrophic *Brevibacterium*, but this was not subsequently confirmed: more recently, methylotrophic species and strains have been isolated of *Bacillus*, *Brevibacterium*, *Rhodococcus*, *Mycobacterium*, *Arthrobacter*, *Micrococcus*, *Amycolatopsis*, *Leifsonia*, and *Gordonia* [17, 18, 24, 29, 45, 67, 117].

3.5.5 Cultivation of Proteobacterial and Gram-Positive Methylotrophs Resident in the Human Body

Several facultatively methylotrophic commensals have been isolated from the human mouth and foot, namely, *Actinobacteria* (*Brevibacterium*, *Gordonia*, *Leifsonia*, *Microbacterium*, *Micrococcus*, *Rhodococcus*) and *Alpha-*, *Beta-*, *and Gammaproteobacteria* (*Achromobacter*, *Klebsiella*, *Methylobacterium*, *Pseudomonas*,

Ralstonia). Some of these grew well on agar or in liquid media on C_1 compounds under normal shake flask conditions [17, 18, 29]. Some isolates from the mouth were, however, unable to grow in normal shake-flask culture (e.g. with rotary shaking at 200 rpm) and grew best with minimal aeration or when grown in static culture with ballotini glass beads in the culture tubes: this method used 10 ml PVM (Sect. 3.3.6) with methanol (50 mM), MMA (20 mM), or formate (25 mM) in statically incubated 25 ml glass or plastic (e.g. 'Sterilin') universal culture tubes, supplemented with 15 g of 2 mm diameter glass beads [29]. Failure to grow in shaken liquid culture was probably due to one or both of oxygen sensitivity and the need to proliferate best on surfaces, which might indicate that their normal growth in the mouth was in biofilms. This criterion needs to be considered when attempting to isolate these human mouth commensals, and indeed isolations from any other habitats in which surface growth may be significant (e.g. water supply pipes).

3.5.6 Methylotrophic Yeasts

An increasing number of methylotrophic yeast species is being demonstrated including species of *Pichia*, *Candida*, and *Hansenula*. These are usually cultured on methanol, commonly with other organic additions including yeast extract and glycerol or co-substrates such as sorbitol [40, 118]. The enzymes for the oxidation and assimilation pathways in their C_1 metabolism differ from those of *Proteobacteria* [38, 39]. The principal interest of methylotrophic yeasts resides in the potential for exploiting large cultivation to yield 'biocommodities' from inexpensive substrates, including synthesis of heterologous protein, enzymes (e.g. α-amylase), and amino acids [40, 119, 120]. Typically 10–90 g methanol l^{-1} was used in laboratory-scale experiments, using bioreactors of 3–150 l capacity, such as the 3 litre Braun CT2-2 bioreactor (**Note 8**). Choi and Kim [40] found 30 g l^{-1} to be optimum for α-amylase production, with 81% of the added methanol being unused at 90 g l^{-1}. Methylotrophic *Pichia pastoris* has also been used for the overproduction of toxin polypeptides encoded by genes inserted from a *Bacillus* [121].

3.5.7 Cultivation of Methylotrophs on an Industrial Scale for Singe-Cell-Protein (SCP) Production

Some bacteria and yeasts have been cultivated on a large scale for commercial production of biomass for protein or secretion of value products such as amino acids. Such operations have used air-lift fermenter systems of up to 2,000 m^3 capacity (two billion litres) [122]. The best known of these was the ICI tower bioreactor of 1,500 m^3 for the mass culture of *Methylophilus methylotrophus* to be used as a SCP feed for animals, with the commercial name of 'Pruteen'. This was a high-quality product (70% cell protein, with a projected output of 70,000 T $year^{-1}$, with a conversion efficiency of methanol to SCP [w/w] of 2:1), and some £100 million was invested in the project in the 1970s. While shown to be technically

feasible, the process was discontinued as it was perceived as not economically viable at the time.

4 Notes

The physicochemical conditions for culture will be determined by the known properties of the organisms studied, or the properties being sought in an enrichment culture, with the following guidelines:

1. Temperature: organisms are normally enriched and isolated either at ambient (20–30°C) temperatures or those reflecting the environment of origin. It is advisable to use a range of temperatures set at ±10°C of those of the origins of the environmental samples, as optimum temperatures may be significantly different from those at the points of isolation. Psychrotrophs isolated from Antarctic samples were enriched and isolated at 12°C, 15°C, and 25°C, but the temperature optima for all were 25–30°C [20, 21]. Isolates obtained from the human body at 37°C had optima around 37°C [17, 18]. Some *Bacillus* species and methylotrophic yeasts are moderately thermophilic, growing optimally at 40–57°C.
2. pH: many methylotrophs isolated to date are neutrophiles, but there are examples of methylotrophic alkaliphilic *Methylobacter* with optima of pH 9–9.5, moderate acidophiles with optima around pH 4.5–5.5, including *Methylocella* and *Methylocapsa*, with a lower limit of pH 4.0–4.5 [15], and extreme acidophiles such as those belonging to the *Verrucomicrobia* which grow optimally at pH 2–2.5 and 55°C [25].
3. Salinity: bacteria obtained from salt lakes and the marine environment may have an obligate requirement for salt, usually as NaCl, but in some environments, NaCl may not be present or may not be the predominant salt, so alternatives may be more appropriate, as determined by the investigator. Marine salt levels are approximately 2.5% (w/v) NaCl, so standard media recipes should be supplemented to enable marine organisms to be isolated (and subsequently maintained). Organisms isolated from tidal rivers where salinity varies might benefit from intermediate levels of NaCl added to the basal medium. Truly halophilic methylotrophs may require highly enhanced levels of NaCl as determined by the investigator.
4. Enhanced CO_2 levels are beneficial or necessary to some methylotrophs especially the methylotrophic autotrophs, such as *Paracoccus versutus*, which does not have the ability to concentrate CO_2 into the cell and is dependent on the ambient concentration available [123]. CO_2 supplementation can be

achieved using Erlenmeyer flasks with Quickfit necks, sealed with 'Subaseal' vaccine stoppers, and injecting sodium bicarbonate solution. For a head space of 250–300 ml, injecting 1 ml 0.5 M $NaHCO_3$ and 0.5 ml 0.5 M H_2SO_4 (to neutralise and release CO_2) is adequate. Chemostat systems need continuous sparging with CO_2 in air, typically at 5% (v/v).

5. Oxygen tension: in chemostat culture of aerobic *Methylobacterium extorquens*, varying dissolved oxygen tension between 1 and 690 mmHg controlled the ratio of methanol oxidised to CO_2, assimilated to cell carbon, and lost as excreted exocellular products [124]. The ratio was constant between 28.5 and 110 mmHg, but outside this range, respiration increased and cell carbon yield decreased. Control of oxygen tension in culture systems is thus crucial in determining the oxidation/assimilation balance in methylotrophs especially if maximum biomass production is required.
6. Anaerobic growth with denitrification: normal basal salts media are supplemented with 25 mM KNO_3 as the respiratory electron acceptor. Cultures can be grown in completely filled sealed containers at the appropriate temperature, and visual observation for growth and denitrification should include the appearance of dinitrogen gas bubbles. Nitrate and nitrite concentrations can be measured during the incubation period by standard analytical procedures. Cultures can also be grown in sealed, shaken flasks, filled with nitrogen or argon, enabling gaseous respiratory oxidants such as N_2O to be used instead of nitrate [125].
7. Volatile, toxic, and unstable substrates (e.g. methanol, volatile methylated sulfur compounds, vitamin solutions) need to be sterilised by membrane filtration: for example, by using 0.2 μm pore-size, solvent-resistant, disposable membrane filters (such as Acrodisc, Michigan, USA). Aqueous solutions can obviously be filtered through standard 0.2 μm pore-size cellulose nitrate membrane filters (e.g. Millipore; Sartorius). Larger volumes of substrates, or solutions, can be filtered using pre-sterilised standard or solvent-resistant syringe filters (e.g. Gilson). Toxic substrates, added in very small volumes, have also been used without prior sterilisation, on the presumption they are already likely to be sterile. Some potentially toxic substrates (e.g. DMS) can only be added in small quantities to avoid culture inhibition, so in liquid culture they are added to cultures in aliquots as low as 1–2 μl every few days. This means a substrate may only reach a final total supplied of 5–10 mM over a period of days or weeks, depending on the growth rates. The vessels used for such cultures need either to have closed lids which are only loosened for the addition of substrate, or to be sealed with red or black rubber vaccine stoppers, or to use

crimp-top containers with a membrane seal, through which fresh substrate can be injected. Some C_1 volatiles are absorbed by red rubber, a problem that can be avoided by using black butyl stoppers (or similar tubing on chemostats) to minimise losses from the cultures. Red 'Subaseals' can also be coated with an impermeable PTFE lubricant and dried before use. For liquid cultures (100 ml volume) in sealed containers, the quantities of substrate required to give suitable concentrations for growth are: 7–18 μl DMS or DMSO gives approximately 1–2.5 mM, with pure DMSO probably being tolerated at 10 mM (71 μl in 100 ml), and 81 μl methanol gives an initial concentration of 20 mM. Growth in sealed flask or bottle cultures can be assessed visually and monitored as increase in optical density (OD) in samples removed by syringe. For growth on agar plates, cultures should be streaked on to agar media lacking substrate. A small piece of sterile filter paper is laid on the inside of the lid when the plate is inverted, and aliquots of the volatile substrate absorbed on the filter paper, allowing it to evaporate into the air space of the plate. Inverted plates are incubated in gas-tight boxes or gas jars to contain and maintain the gaseous atmosphere. Additions of extra substrate can be made every few days as judged appropriate by visual observation of growth on the agar. Some substrates can be foul-smelling as well as toxic and the handling of materials such as MT, DMS, and DMDS must be done in fume/extractor hoods, using sealed vessels, and observing manufacturers' safety guidelines, as well as those of the laboratory/institution where the work is done. The same techniques can be used for any volatile substrates, including methanol and DMSO. High-purity DMS and DMSO are commercially available in argon-filled, sealed, crimp-top bottles, and aliquots should be removed only through the septum seal, using a Hamilton microsyringe (Hamilton Company, Nevada, USA) and fine needles.

8. Growth-limiting nutrients: in relation to large scale cultivation in bioreactors, applicable also to continuous chemostat or fed-batch cultivation, the composition of the media used is a key consideration. Some of the media described in this chapter contain relatively low concentrations of essential nutrients, such as nitrogen, which could potentially become growth limiting: the *Paracoccus versutus* medium, for example, contains 0.4 g $NH_4Cl\ l^{-1}$. This provides 0.1 g $N\ l^{-1}$ which, at a cell content of 10% (w/w) nitrogen, is sufficient to support biomass production of 1 g dry weight of bacteria per litre. This is adequate for low yield bacteria such as chemolithoautotrophs but could become limiting for growth on some C_1 compounds. The AMS medium is similar with only 0.5 g $NH_4Cl\ l^{-1}$. For

example, methanol at 1% (w/v; 313 mM) provides 3.75 g carbon l^{-1}; if biomass production is 50% efficient, this allows production of about 1.9 g cell carbon l^{-1}; with cell carbon at 50% of the dry weight, the methanol could support growth of about 3.8 g dry weight l^{-1}, but would require about 1.5 g $NH_4Cl\,l^{-1}$. While some reports show use of methanol at such a high concentration, most papers report use of much lower concentrations, in the range 10–50 mM methanol, for which NH_4Cl at 0.4 g l^{-1} is sufficient. Mass culture with high concentrations of substrate or batch systems fed with successive inputs of substrate will require much more N to be supplied: in the Pruteen bioreactor, for example, adequate nitrogen was provided by an input of pure ammonia. For normal laboratory culture, nitrogen should probably ideally be supplied at 0.8–1.6 g $NH_4Cl\,l^{-1}$ or proportionate amounts of other nitrogen sources such as ammonium sulfate or potassium nitrate.

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Cultivation of Methanotrophs

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Abstract

Aerobic methanotrophs are metabolically unique bacteria that are able to utilize methane as a sole source of energy. The selective approach to enrich methanotrophs therefore employs mineral media with methane added as a growth substrate. The composition of a mineral medium and the incubation conditions strongly determine the outcome of the enrichment procedure. Adjusting medium composition to mirror the conditions of a natural habitat is a key to isolating environmentally relevant organisms. Methane-oxidizing enrichments typically contain many non-methanotrophic bacteria growing on by-products of methanotrophs or on medium contaminants, so obtaining isolated cultures is a time-consuming process that involves repeated series of plating on solid media, dilution–extinction in liquid media, and other purification procedures. Purity tests represent an important component of the isolation procedure. Final steps include registration of growth dynamics on methane and molecular identification of isolates.

Keywords: Growth on methane, Methane-oxidizing enrichment cultures, Methanotrophic bacteria, Mineral media, Purification procedures

1 Introduction

Aerobic methanotrophs are a unique subset of methylotrophic bacteria that can utilize methane (CH_4) as a sole energy source. A defining characteristic of these organisms is the use of methane monooxygenase (MMO) enzymes to catalyze the oxidation of methane to methanol. The selective approach to enrich and cultivate methanotrophs, therefore, implies the use of mineral media with methane as a growth substrate.

The history of methanotroph cultivation dates back to 1906, when the first methanotrophic bacterium was isolated by Söhngen and named *Bacillus methanicus* (now known as *Methylomonas methanica*) [1]. Since that time, the number and diversity of cultured methanotrophs have gradually increased. At present, methanotrophic capabilities are recognized in members of the bacterial phyla *Proteobacteria*, *Verrucomicrobia*, and the candidate division NC10 [2]. Most of the currently available knowledge is specific to methanotrophic *Proteobacteria*. These microorganisms

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affiliate with the classes *Gammaproteobacteria* (type I methanotrophs) and *Alphaproteobacteria* (type II methanotrophs) and occur in a wide range of habitats where both methane and oxygen are available [3]. Nearly all methanotrophic bacteria that are now available in pure cultures belong to the *Proteobacteria*, while only a very few strains represent the *Verrucomicrobia*. The latter were only recently discovered [4] and appear to be restricted to acidic geothermal environments [5]. Methanotrophic representatives of the candidate phylum NC10 occur in anoxic habitats and have an intra-aerobic pathway of CH_4 oxidation [6]. These bacteria have not yet been obtained in pure cultures and are not further discussed in this chapter.

The seminal work on methanotroph cultivation belongs to Whittenbury and colleagues [7], who isolated more than one hundred strains of these bacteria from various environmental samples. The approach proposed by these authors and the two mineral media, nitrate and ammonium mineral salts (NMS and AMS, respectively), are still widely used for culturing methanotrophs today. These media, however, select for neutrophiles and are most suitable for isolation of methanotrophs from freshwater environments and salt-free terrestrial habitats with near-neutral pH. Further molecular research into methanotroph diversity has indicated that alkaline and acidic as well as saline environments are also inhabited by methanotrophic bacteria, which cannot be cultured using NMS and AMS media. Successful isolation of these bacteria in pure cultures was achieved by adjusting medium composition to mirror the conditions of a respective natural habitat. Thus, acidophilic methanotrophs that inhabit acidic, nutrient-poor northern wetlands were isolated using dilute, mildly acidic (pH 4–5) media with a very low salt content (20–200 mg L^{-1}) and low buffering capacity [8–10]. Extremely acidophilic *Verrucomicrobia* methanotrophs were obtained from volcanic habitats in mineral salt media strongly acidified (pH 1–3) with H_2SO_4 [4]. Cultivation of halotolerant and halophilic methanotrophs, by contrast, involved the use of media with the total salt content of 50–100 g L^{-1} [11, 12]. Isolation of alkaliphilic methanotrophs from soda lakes was achieved on mineral media adjusted to pH 9–10 with a mixture of $NaHCO_3/Na_2CO_3$ [11, 13]. Though many different media for methanotroph cultivation have been proposed to date (Table 1), their compositions do not cover the whole spectrum of conditions in natural environments. Choosing the proper medium or designing a new one, therefore, remains one of the most important tasks in methanotroph cultivation.

The choice of a proper medium, however, is only a first step to success in isolating methanotrophs from a particular environment. Methane-oxidizing enrichment cultures represent complex microbial consortia in which methanotrophic bacteria are tightly integrated with methylotrophic and heterotrophic satellite bacteria.

Table 1
Mineral media used for cultivation of methanotrophic bacteria

Medium (reference)	Composition (g per liter distilled water)	Target group of methanotrophs
Nitrate mineral salts (NMS) medium [7, 14]	KNO_3, 1; $MgSO_4 \times 7H_2O$, 1; $Na_2HPO_4 \times 12\ H_2O$, 0.717; KH_2PO_4, 0.272; $CaCl_2 \times 6H_2O$, 0.2; ferric ammonium EDTA, 0.004; 0.1% (v/v) of trace element solution no. 1 (Table 2); pH 6.8. For marine methanotrophs, add 1.5–3.5% NaCl or artificial sea salts and 1 mL of filter-sterilized vitamin solution 1 (Table 3)	Neutrophiles from freshwater environments, marine methanotrophs
Ammonium mineral salts (AMS) medium [7, 14]	NH_4Cl, 0.5; $MgSO_4 \times 7H_2O$, 1; $Na_2HPO_4 \times 12\ H_2O$, 0.717; KH_2PO_4, 0.272; $CaCl_2 \times 6H_2O$, 0.2; ferric ammonium EDTA, 0.005; 0.1% (v/v) of trace element solution no. 1 (Table 2); pH 6.8.	Neutrophiles from freshwater environments
Medium 10 [15]	NH_4Cl, 0.5; $MgSO_4 \times 7H_2O$, 0.1; $Na_2HPO_4 \times 12\ H_2O$, 0.7; KH_2PO_4, 0.3; $CaCl_2 \times 6H_2O$, 0.1; $FeSO_4 \times 7H_2O$, 0.005; 0.1% (v/v) of trace element solution no. 2 (Table 2); pH 7.0	Neutrophiles from freshwater environments
Dilute nitrate mineral salts (DNMS) medium [19]	This is NMS medium diluted 1:5 with distilled H_2O and containing 1 mM of NaH_2PO_4-Na_2HPO_4 buffer corresponding to a certain pH (5.5–7.0).	Mild acidophiles and neutrophiles from freshwater and salt-free terrestrial environments
Medium M2 [8]	KNO_3, 0.25; KH_2PO_4, 0.1; $MgSO_4 \times 7H_2O$, 0.05; $CaCl_2 \times 2H_2O$, 0.01; NaCl, 0.02; 0.1% (v/v) of trace element solution no. 1 (Table 2); pH 5.5.	Methanotrophs from freshwater wetlands and mildly acidic soils
Medium N [10]	KH_2PO_4, 0.002; KNO_3, 0.001; NH_4Cl, 0.008; NaCl, 0.003; $CaCl_2 \times 2H_2O$, 0.018; $MgSO_4 \times 7H_2O$, 0.010; Na_2SiO_3, 0.002; $AlCl_3$, 0.001; 0.2% (v/v) of trace element solution no. 1 (Table 2).	Mild acidophiles from ombrotrophic wetlands
Medium 3.9C10.2 [5]	NH_4Cl, 0.2; KH_2PO_4, 0.05; $MgSO_4 \times 7H_2O$, 0.02; $CaCl_2 \times 6H2O$, 0.01; FeEDTA powder, 0.005; 0.3% (v/v) of trace element solution no. 3 (Table 2); pH 3.9.	Acidophiles from geothermal soils

(continued)

Table 1
(continued)

Medium (reference)	Composition (g per liter distilled water)	Target group of methanotrophs
Medium S [12]	NH_4NO_3, 0.5; $Na_2HPO_4 \times 2H_2O$, 0.5; KH_2PO_4, 0.1; $FeSO_4 \times 7H_2O$, 0.005; NaCl, 66.0; Na_2SO_4, 10.45; Na_2CO_3, 0.10; $NaHCO_3$, 0.06; NaBr, 0.28; $MgCl_2$, 20.89; $CaCl_2$, 2.21; 0.1% (v/v) of trace element solution no. 1 (Table 2); pH 7.5	Neutrophilic halophiles
Medium SL [13]	Na_2CO_3, 23; $NaHCO_3$, 7; NaCl, 5; K_2HPO_4, 0.5; KNO_3, 0.5; $MgSO_4 \times 7H_2O$, 0.12; 0.1% (v/v) of trace element solution No 4 (Table 2); pH 10.1	Alkaliphiles

Table 2
Trace element stock solutions

No. (reference)	Composition (g per liter distilled water)	Applicability
No. 1 [14]	Na_2EDTA, 0.5; $FeSO_4 \times 7H_2O$, 0.2; H_3BO_3, 0.03; $ZnSO_4 \times 7H_2O$, 0.01; $MnCl_2 \times 4H_2O$, 0.003; $CoCl_2 \times 6H_2O$, 0.02; $CuSO_4 \times 5H_2O$, 0.03; $NiCl_2 \times 6H_2O$, 0.002, $Na_2MoO_4 \times 2H_2O$, 0.003	Most extant methanotrophs (Note that the Cu concentration (critical for pMMO) is lower than in other solutions and is often increased 10×)
No. 2 [15]	$ZnSO_4 \times 7H_2O$, 0.44; $CuSO_4 \times 5H_2O$, 0.22; $MnSO_4 \times 2H_2O$, 0.17; $Na_2MoO_4 \times 2H_2O$, 0.06; H_3BO_3, 0.10; $CoCl_2 \times 6H_2O$, 0.08	Most extant methanotrophs
No. 3 ([16], improved based on [17])	Na_2EDTA, 2.06; $FeSO_4 \times 7H_2O$, 1.54; $ZnSO_4 \times 7H_2O$, 0.44; $CuSO_4 \times 5H_2O$, 0.20; $MnCl_2 \times 4H_2O$, 0.19; $Na_2MoO_4 \times 2H_2O$, 0.06; H_3BO_3, 0.10; $CoCl_2 \times 6H_2O$, 0.08., $CeCl_3 \times 7H_2O$ 0.1; $LaC1_3 \times 7H_2O$ 0.1	Methanotrophs from geothermal soils
No. 4 [13]	Na_2EDTA, 5.0; $FeSO_4 \times 7H_2O$, 2.0; $CuSO_4 \times 5H_2O$, 0.1; $ZnSO_4 \times 7H_2O$, 0.1; $MnCl_2 \times 4H_2O$, 0.03; $CoCl_2 \times 6H_2O$, 0.2; $NiCl_2 \times 6H_2O$, 0.02; $Na_2MoO_4 \times 2H_2O$, 0.03	Alkaliphilic methanotrophs

Table 3
Vitamin stock solution

No. (reference)	Composition (g per liter distilled water)	Applicability
No. 1 [18]	Biotin, 0.02; folic acid, 0.02; thiamine HC1, 0.05; calcium pantothenate, 0.05; B12, 0.001; riboflavin, 0.05; nicotinamide, 0.05 To be added with sterile filter after autoclaving the medium	Marine methanotrophs, may also be helpful with terrestrial methanotrophs

These thrive on contaminants of the media (esp. of agar), and on metabolites excreted by methanotrophs, including organic acids like formate, acetate, succinate, and lactate [20, 21]; methanol [22]; formaldehyde [23]; exopolysaccharides [24, 25]; and cell lysis products such as amino acids and nucleic acids [20, 26]. These satellite bacteria may directly improve growth of the methanotrophs through removal of inhibitory products or through other interactions such as the production of siderophores. Separation of methanotroph cells from these associated bacteria is the most challenging and time-consuming part of the isolation procedure. The desired purification is usually achieved by repeated rounds of serial dilutions in a liquid medium followed by spread plating onto a solid medium. Final steps of the isolation procedure include thorough examination of the methanotrophic cultures for purity, registration of their growth dynamics on methane, and molecular identification.

In summary, despite accumulating knowledge, isolation and cultivation of new taxa of methanotrophs remains a difficult task. Various molecular techniques can aid different stages of the isolation procedure, starting with detection of methanotroph cells in enrichment cultures and ending up with tests for culture purity. This chapter describes the most common techniques used in methanotroph cultivation as well as some optional approaches that may be helpful.

2 Materials

2.1 Growth Media

2.1.1 Mineral media used for methanotroph cultivation: *see* Table 1 and **Note 1.** Chemicals needed to prepare these media include KNO_3, NH_4Cl, $MgSO_4 \times 7H_2O$, $Na_2HPO_4 \times 12\ H_2O$, NaH_2PO_4, KH_2PO_4, $CaCl_2 \times 6\ H_2O$, and NaCl (Sigma, http://sigmaaldrich.com).

2.1.2 Trace elements stock solutions: *see* Table 2. Chemicals needed include EDTA, $FeSO_4 \times 7H_2O$, H_3BO_3, $ZnSO_4 \times 7H_2O$, $MnCl_2 \times 4H_2O$, $MnSO_4 \times 2H_2O$, $CoCl_2 \times 6\ H_2O$, $CuSO_4 \times 5H_2O$, $NiCl_2 \times 6H_2O$, and $Na_2MoO_4 \times 2H_2O$. In the case of *Verrucomicrobia* methanotrophs

in geothermal environments, $LaCl_3 \times 7H_2O$ and $CeCl_3 \times 7H_2O$ will also be required (*see* **Note 2**).

2.1.3 Vitamin solutions can improve the growth of some strains (*see* Table 3).

2.1.4 Complex organic media for testing the presence of heterotrophic satellites in methanotrophic cultures: Luria-Bertani agar, R2A, or nutrient Agar (Difco, http://www.bd.com) and several individual multicarbon compounds, such as glucose, fructose, or sucrose, and yeast extract.

2.1.5 Solidifying agents: agar (Difco, http://www.bd.com), noble agar (Difco, http://www.bd.com), agarose (Difco, http://www.bd.com), gellan gum (Gel-Gro; ICN Biomedicals, http://www.mpbio.com), or Phytagel (Sigma).

2.1.6 Gases: CH_4 and CO_2 (*see* **Note 3**).

2.2 Cultivation Flasks and Incubation Chambers

2.2.1 Serum bottles (total volume 30–100 mL) for cultivation in liquid media (Sigma, http://sigmaaldrich.com or Wheaton http://www.fishersci.com).

2.2.2 Silicone or gray butyl rubber stoppers, aluminum crimp seals, and a bottle crimper (Sigma, http://sigmaaldrich.com).

2.2.3 Alternatively to 2.2.1 and 2.2.2, screw-capped serum flasks with perforated caps and silicone septa/stoppers can be used (Sigma, http://sigmaaldrich.com).

2.2.4 Glass desiccator fitted with a valve for gas exchange (SciLabware, http://www.scilabware.com), gassable "anaerobic" jar such as an Oxoid HP0011A with Schrader valves (http://www.thermoscientific.com), or any kind of a gas-tight jar for incubation of Petri plates under a controlled atmosphere.

2.2.5 MILLEX® syringe filter units, 0.22 μm (Millipore, www.millipore.com).

2.3 Microscopy

2.3.1 Slides for phase-contrast microscopy and glass coverslips (Sigma, http://sigmaaldrich.com).

2.3.2 Teflon-coated diagnostic slides with 8 wells for whole-cell hybridization (Thermo Scientific, http://www.thermoscientific.com).

2.4 Whole-Cell Hybridization with Fluorescent Probes

2.4.1 Paraformaldehyde and formamide (Sigma, http://sigmaaldrich.com).

2.4.2 50-mL polypropylene screw-top Falcon tubes (Corning, http://www.corning.com).

2.4.3 Fluorescently labeled 16S rRNA-targeted oligonucleotide probes. The equimolar mixture of probes M84

(5′-CCACTCGTCAGCGCCCGA-3′) and M705 (5′-CTGGTGTTCCTTCAGATC-3′) is used for specific detection of type I methanotrophs, while the probe M450 (5′-ATCCAGGTACCGTCATTATC-3′) is suitable for detection of type II methanotrophs (*see* **Note 4**). The probes are synthesized and labeled commercially (ATDBio, http://www.atdbio.com).

2.5 Molecular Identification

2.5.1 Primers targeting MMO-encoding genes: A189f (5′-GGNGACTGGGACTTCTGG-3′) and A682r (5′-GAASGCNGAGAAGAASGC-3′) or mb661 (5′-CCGGMGCAACGTCYTTACC-3′) for amplification of the *pmoA* gene from proteobacterial methanotrophs [27]; V170f (5′-GGATWGATTGGAAAGATMG-3′) and V631b (5′-GCAAARCTYCTCATYGTWCC-3′) for amplification of this gene from thermophilic verrucomicrobial methanotrophs [28] or LVpmoAf (5′-GGRTKGACTGGAAAGAYCG-3′) and LVpmoAb (5′-GCGAARCTYCGCATCG TTCC-3′) for amplification of this gene from mesophilic verrucomicrobial methanotrophs [5].

2.5.2 Primers mmoXA-166f (5′-ACCAAGGARCARTTCAAG-3′) and mmoXD-1402r (5′-TGGCACTCRTARCGCTC-3′) for amplification of the *mmoX* gene [29] (*see* also **Note 5**).

2.5.3 PCR Master Mix (Promega, http://www.promega.com) for amplification of *pmoA* or *mmoX* gene fragments.

2.5.4 PCR cleanup kit (Promega, http://www.promega.com).

2.6 General Buffers and Solutions

2.6.1 Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g NaH_2PO_4, per 1 L, and pH 7.0.

2.6.2 Absolute ethanol as well as 50, 70, and 80% (v/v) ethanol.

2.6.3 5 M NaCl: dissolve 292 g NaCl in 1 L distilled water.

2.6.4 1 M Tris/HCl, pH 7.2: dissolve 121 g Tris base in 800 mL distilled water, adjust to pH 7.2 with concentrated HCl, and mix and add water to 1 L.

2.6.5 0.5 M EDTA: dissolve 186.1 g $Na_2EDTA \times 2H_2O$ in 700 mL distilled water, adjust pH to 8.0 with 10 M NaOH, and add water to 1 L.

2.6.6 10% (w/v) sodium dodecyl sulfate (SDS) (all reagents for buffers and stock solutions were purchased in Sigma; http://sigmaaldrich.com).

3 Methods

It is possible to start isolation efforts by adding environmental samples, or serial dilutions thereof, directly to solid mineral salt medium on Petri plates and incubating these under a methane-enriched atmosphere. This is often successful with communities where methanotrophs are abundant [5, 19]. However, with the exception of methane-rich environments, methanotrophic bacteria constitute only a minor part (<1% of total bacterial cells) of the indigenous microbial community in most habitats, and therefore, an enrichment procedure is often carried out before this step. Two approaches are common: 1) the sample of interest is incubated with methane under in situ conditions, or 2) the sample of interest is used to inoculate a liquid mineral medium and is incubated under certain conditions in the laboratory. The first approach is clearly more suitable for isolation of environmentally relevant organisms. Actual incubation in situ with a gaseous substrate is very difficult. However, raw environmental samples such as soil can be incubated in the lab under conditions (esp. temperature) that mimic the natural site. One potentially useful modification of this approach is cultivation of methanotrophs on polycarbonate membranes, which are placed on top of a non-sterile environmental sample such as soil and incubated in an atmosphere containing methane in air [30]. Nutrients from the soil can diffuse through the membrane and stimulate the growth of the methanotrophs on the membrane surface.

Another approach that mimics the in situ gas profiles of many methanotroph habitats is growing methanotrophs in counter gradients of methane and O_2 [23, 31]. In this approach, a column containing semisolid mineral salt medium mixed with an environmental sample is fed with methane from below and air from above, mimicking the situation in many wetland and aquatic environments where methane is produced in deep anoxic sediments and diffuses upwards to the oxic boundary. As methanotroph growth progresses in the columns, a band of optimal growth occurs where methane and O_2 gradients cross and both gases are depleted. This system can mimic low O_2 and methane concentrations in the field and also allow toxic by-products of methanotrophy to diffuse away from the cells [23].

These alternative approaches, while promising, have not yet been widely used, and obtaining enrichment cultures on artificial mineral media with simple batch and plate cultivation techniques remains the most common way to isolate methanotrophs.

3.1 Enrichment Cultures

Since different media select for different methanotrophic bacteria, it is recommended to choose (or design) the medium whose composition best mirrors the conditions of a natural habitat.

Prepare the most suitable liquid mineral medium from a list of media given in Table 1 (*see* **Note 6**).

1. Fill serum flasks to 20% capacity with the liquid medium, sterilize, and inoculate with a small amount of sample.
2. Seal the flasks with rubber stoppers (*see* **Note 7**) and add methane (10–30%, vol/vol) and CO_2 (5% vol/vol) to the headspace using a syringe and a sterile MILLEX® syringe filter unit (0.22 μm).
3. Incubate flasks on a rotary shaker (100–150 rpm) or in static conditions (*see* **Note 8**) at an optimal growth temperature.

The enrichment usually takes one to several weeks, depending on the sample and incubation conditions used. Turbidity or, in some cases, a pellicle of growth in the flasks indicates development of target bacteria. Suspensions sampled from these enrichment cultures can be examined using phase-contrast microscopy (due to the presence of intracytoplasmic membranes, cells of methanotrophs usually look somewhat darker than cells of non-methanotrophic satellites). Molecular detection of the *pmoA* gene (*see* Sect. 3.8) or whole-cell hybridization with methanotroph-specific 16S rRNA-targeted probes can be applied at this stage (*see* Sect. 3.5). Further isolation of methanotrophs in pure cultures can be done by either serial dilutions in a liquid medium or by spread plating onto a solid medium.

3.2 Serial Dilutions

1. Prepare 5–7 small (30–50 mL volume) serum flasks filled to 20% capacity with the liquid medium.
2. Inoculate the first flask with an aliquot (0.5 mL) of a cell suspension taken from the enrichment culture and prepare serial dilutions in tenfold steps in remaining flasks.
3. Seal the flasks and add methane (10–30%, vol/vol) and CO_2 (5% vol/vol) to the headspace by using a syringe and a sterile filter (0.22 μm). Incubate flasks on a rotary shaker (100–150 rpm) or in static conditions at an optimal growth temperature for 2–3 weeks.
4. Examine the culture suspensions from the flasks with highest dilutions in which growth is visible by phase-contrast microscopy. Select the culture containing a large proportion of morphologically uniform cells and repeat the process of serial dilutions until a culture consisting of morphologically uniform cells is obtained.

3.3 Spread Plating

1. Prepare plates with a mineral medium of choice solidified with either agar (1.0–2.0%, w/v) or gellan gum (1%, w/v) (*see* **Notes 9** and **10**). To decrease growth of non-methanotrophic

bacteria on agar contaminants, noble agar or agarose may be used instead of standard agar.

2. Spread an aliquot (0.05–0.2 mL) of a cell suspension taken from the enrichment culture or one of the flasks with serial dilutions onto the plates.
3. Incubate plates in a closed vessel (a glass desiccator or gastight jar) containing air supplemented with 10–30% CH_4 and 5% CO_2 for 2–4 weeks.
4. Select colonies of interest for verification of methanotroph identity and restreaking (*see* **Note 11**). A stereomicroscope may be employed to aid in selecting individual methanotroph colonies apart from neighboring non-methanotroph colonies. Repeat the spread-plating procedure until uniform pure colonies are obtained.

3.4 Alternative Purification Procedures

In some cases, the above-described serial dilutions in a liquid medium or spread plating onto a solid medium can result in a methanotrophic pure culture. In most cases, however, repeated series of both approaches are required to purify a methanotroph from non-methanotrophic satellites. This is the most time-consuming step in methanotroph cultivation, which may take up to several years. Additional approaches that may be helpful for purification of particular methanotroph types are listed below:

1. Methanotrophs with large cells (*see* **Note 12**) can potentially be purified from non-methanotrophic satellites by filtration through 0.6–1 μm Millipore filters or by differential centrifugation at 2,000*g* for 10 min [11].
2. For purification of fast-growing methanotrophs (*see* **Note 13**), serial dilutions in 96-well microtiter plates can be efficient [32]. This is also a miniaturized approach for optimization of a medium composition.

3.5 Purity Tests

Routine tests for the presence of heterotrophic satellites in apparently pure methanotrophic cultures are plating on (1) complex organic media (standard undiluted and tenfold-diluted Luria-Bertani agar, R2A, or nutrient agar; see Sect. 2.1.4) and (2) the same mineral media as used for methanotroph isolation supplemented with 0.05% (w/v) glucose, fructose, or sucrose and 0.005% (w/v) yeast extract:

1. Spread an aliquot (0.05–0.1 mL) of a methanotroph culture onto the plates with nutrient media and incubate without methane for 2–4 weeks.

No growth should be observed on the plates, except in the rare case of facultative methanotrophs like *Methylocella* that may grow weakly on complex media like R2A [33]. Some

non-methanotrophic satellites (e.g., facultative methylotrophs) may not develop on these media and may escape detection. More thorough and complex tests for culture purity can be performed as described in [33].

3.6 Whole-Cell Hybridization with Methanotroph-Specific Fluorescent Probes

The use of whole-cell hybridization with 16S rRNA-targeted, fluorescently labeled oligonucleotide probes is a highly effective way to trace the development of methanotroph cells in enrichment cultures as well as to examine the obtained isolates for contamination. This is an optional procedure, which can be added as the final step in any of the above-described protocols 3.1–3.4 or serve as an additional purity test in Sect. 3.5:

1. Collect cells from the culture of interest and resuspend in 0.5 mL of PBS.
2. Mix cell suspension with 1.5 mL of 4% (w/v) freshly prepared paraformaldehyde solution and incubate for 1 h at room temperature.
3. Collect cells by centrifugation (6,600*g* for 1 min) and wash twice with PBS to ensure removal of paraformaldehyde. Resuspend the resulting pellet in 0.5 mL of 50% ethanol–PBS (v/v).
4. Spread 1–2 μL of the fixed cell suspension on each well of a Teflon-coated slide, air-dry, and dehydrate by successive passages through an ethanol series (50, 80, and 100% (v/v)) for 3 min each.
5. Prepare a hybridization chamber of a 50-mL polypropylene screw-top Falcon tube containing a slip of a filter paper soaked in hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 7.2, 0.01% SDS, and formamide concentration corresponding to the probe stringency conditions). Equilibrate the chamber for at least 30 min at the hybridization temperature.
6. Place a 9-mL aliquot of hybridization buffer on each spot of fixed cells. Transfer the slide to the equilibrated chamber and prehybridize it for 15 min. Add 1 mL of fluorescent probe (50 ng mL^{-1} solution in double-distilled water) to each spot and return the slide to the hybridization chamber for 1–1.5 h.
7. Wash the slide at the hybridization temperature for 10 min in washing buffer (20 mM Tris/HCl, 0.01% SDS, and NaCl concentration corresponding to the probe stringency conditions) and then rinse with distilled water and air-dry.
8. Mount each well of the slide with a drop of glycerol, cover with a coverslip, and view with an epifluorescent microscope equipped with the filters for corresponding fluorophore.

3.7 Measuring Growth Dynamics on Methane

These experiments are required to confirm the ability of the isolated strain to develop on methane and are usually conducted using liquid cultures. An increase in optical density (OD_{600}) should be accompanied by a decline in CH_4 mixing ratio of the headspace (*see* **Note 14**), while no growth should observed in the same medium in the absence of methane. Methane concentration is measured using a gas chromatograph equipped with a flame ionization detector and an appropriate column (e.g., Porapak Q, Mol Sieve 5A). Alternatively, a thermal conductivity detector and an appropriate column may be used. The optical density is measured on a spectrophotometer:

1. Prepare 100-mL serum flasks filled to 20% capacity with the liquid mineral medium. Inoculate the flasks with cells of a target strain to achieve an initial OD_{600} of 0.01–0.03.
2. Seal the flasks and add methane (10%, vol/vol) to the headspace by using a syringe and a sterile filter (0.22 μm). Include uninoculated controls of the medium as blanks to control for methane leakage and sterility control, and also include inoculated medium with no added methane. Incubate flasks on a rotary shaker (100–150 rpm) or in static conditions at an optimal growth temperature.
3. Take gas samples and culture aliquots once every 1–2 days for determination of methane concentrations and OD_{600}.

3.8 Molecular Identification

All aerobic methanotrophs contain one or both of two potential MMO enzymes, particulate and/or soluble MMO. Particulate MMO (pMMO) is encoded by a *pmoCAB* operon, while soluble MMO (sMMO) is encoded by a more complex set of genes including *mmoXYBZDC*. The *pmoA* and *mmoX* genes encoding the β-subunit of pMMO and the α-subunit of the sMMO hydroxylase, respectively, are commonly used as functional markers for these bacteria. Determination of either *pmoA* or *mmoX* gene sequences from a new isolate in addition to determination of 16S rRNA gene sequence is an obligate requirement for methanotroph identification. Colony PCR of the *pmoA* and *mmoX* genes may also be used as an early screen of a methanotroph enrichment to identify target colonies (*see* also **Note 15**):

1. Use the primers A189 + A682 or A189 + mb661 to amplify the *pmoA* gene fragment. The PCR conditions are: an initial denaturation step of 96°C for 4 min, followed by 30 cycles of 92°C for 1 min, 56°C for 1 min, 72°C for 45 s, and a final extension of 5 min at 72°C. The expected PCR product should be approx. 525 bp in length (*see* **Note 16**).
2. Use the primers mmoXA-166f + mmoXD-1401r to amplify the *mmoX* gene fragment The PCR conditions are: an initial denaturation step of 94°C for 30s, followed by 30 cycles of 92°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final

extension of 5 min at 72°C. The expected PCR product should be approx. 1,230 bp in length.

3. In case of a verrucomicrobial methanotrophs, the primer set V170f + V631b was designed to target two of the three *pmoA* genes from known thermophilic verrucomicrobial methanotrophs [28], and the primer set LVpmoAf + and LVpmoAb was designed to target the genes from known mesophilic methanotrophs [5]. However, due to the small *pmoA* databases for this group, neither of these may be useful for new isolates, and 16S rRNA-based analysis may be preferable. Genes for MMO in the known *Verrucomicrobia* methanotrophs have to date all been identified based on whole-genome sequencing [4].
4. Purify the resulting PCR product using a PCR cleanup kit and sequence it.

4 Notes

1. The choice of a proper medium depends on the characteristics of a sample used for methanotroph isolation. Medium additions such as soil extracts, vitamin solutions (*see* Table 3), or yeast extract may be used as well, but note that these additions may support the growth of non-methanotrophic heterotrophic bacteria.
2. It has been shown that growth of *Verrucomicrobia* methanotrophs from geothermal environments is strictly dependent on the presence of lanthanides, a group of rare earth elements such as lanthanum, cerium, praseodymium, and neodymium [5, 17].
3. Growth of many methanotrophs is stimulated in the presence of CO_2, which is involved in primary and intermediate metabolism of these bacteria. In type I methanotrophs, 5–15% of the carbon in cell biomass is derived from CO_2, while in type II methanotrophs, the proportion of CO_2-derived carbon is up to 50% [34]. Methanotrophic *Verrucomicrobia* are autotrophs, which use the Calvin–Benson–Bassham (CBB) cycle for CO_2 fixation [35]. Some type I methanotrophs (*Methylococcus* and *Methylocaldum*) also possess CBB cycle.
4. Please note that the probes M84 + M705 do not detect members of the genera *Methylothermus*, *Methylohalobius*, and *Methylomarinovum*, while the probe M450 does not target methanotrophic representatives of the *Beijerinckiaceae* (the genera *Methylocapsa*, *Methylocella*, and *Methyloferula*). The extended set of probes for detection of methanotrophic bacteria can be found in [9].
5. Besides the most widely used pairs of primers listed in Materials, a wide variety of primers targeting *pmoA* and *mmoX* of different proteobacterial methanotroph groups is available [27, 33].

6. In order to avoid precipitation of some media ingredients during sterilization, phosphates and nitrogen sources are usually prepared as separate stock solutions and are added to the medium after sterilization.
7. During sterilization, some kinds of butyl rubber stoppers seem to emit volatile compound(s), which strongly inhibit growth of methanotrophs. One particular example is black butyl rubber stoppers produced by Rabo B.V. (Netherlands). To minimize this effect, sterilize stoppers separately from flasks with growth media. The use of twice boiled stoppers (in distilled water for at least 30 min) is an alternative option.
8. Incubations on a shaker or in static conditions select for different methanotrophic bacteria [36]. When using nitrogen-free media for isolation of nitrogen-fixing methanotrophs, incubation in static conditions is recommended because dinitrogenase of these bacteria is sensitive to oxygen.
9. For the preparation of gellan plates, prepare the medium in 500 mL of water and adjust to the desired pH. Prepare a separate 500-mL solution containing 0.5 g of $MgSO_4 \times 7 H_2O$ and slowly add 5 g of gellan. Do not adjust the pH of the gellan solution. Autoclave the two solutions separately and combine immediately before pouring plates.
10. Contamination of plates with fungi is common because of the long incubation times required (typically >4 weeks). Antifungal agents such as cycloheximide and nystatin can be added to final concentrations of around 50 μg mL^{-1} with a sterile syringe and filter after autoclaving the medium.
11. Many colonies that appear on solid mineral media after incubation under methane belong to non-methanotrophs. These may utilize the polymers used as gelling agents, metabolites produced by a nearby growing methanotroph, or contaminants of the media components. Typical examples of such non-methanotrophic bacteria are members of the genera *Burkholderia* and *Bradyrhizobium*. Methanotrophs are usually the largest colonies on the plates, and the other bacterial typically form small, flat, often nearly transparent colonies. Ways to select the colonies of methanotrophs include (1) examination of cells using phase-contrast microscopy (methanotrophs are usually >1 μm in diameter and appear darker than satellite bacteria due to the presence of intercellular membranes), (2) whole-cell hybridization with methanotroph-specific 16S rRNA-targeted probes, (3) colony PCR with *pmoA*-specific primers, and (4) transfer of individual colonies to flasks with a liquid medium and cultivation with methane as the only growth substrate.
12. Representatives with relatively large cells (1.5–3 μm) are most common among type I (gammaproteobacterial)

methanotrophs. The examples are members of the genera *Methylobacter*, *Methylococcus*, *Methylovulum*, *Methylomonas*, and *Methylosoma*. With the only exception of *Methylosinus trichosporium*, the cells of type II (alphaproteobacterial) methanotrophs are usually smaller in size.

13. The potential of this approach was demonstrated using fast-growing methanotrophs of the genus *Methylomonas*. It is not applicable for purification of slow-growing methane oxidizers.
14. Methanotrophs possessing particulate MMO show good growth in these experiments with OD_{600} reaching 0.8–1.5 within 3–6 days. By contrast, methanotrophs that possess only a soluble MMO may require up to 2–3 weeks until the cultures reach OD_{600} of 0.2–0.3. Measuring methane decline via GC is critical, and some false reports of "unusual" methanotrophic bacteria exist in the literature that failed to demonstrate methane oxidation.
15. Please note that the use of standard assays can also result in exclusion of novel methanotrophs, not always but mostly belonging to distinct, as-yet-undescribed groups.
16. Both primer sets are considered universal, although neither amplifies genes from *Verrucomicrobia* methanotrophs, and *pmoA* genes from certain species of proteobacteria may be poorly amplified. The A189 and A682 are considered better for the *Alphaproteobacteria*, while the A189 + mb661 is considered better for *Gammaproteobacteria*. For detailed molecular procedures for detection of MMO-encoding genes, see [27].

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Cultivation of Environmental Bacterial Communities as Multispecies Biofilms

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Abstract

Microbes play an important role in the biogeochemistry of hydrocarbons and are frequently studied for potential applications in hydrocarbon biotechnologies. The empirical study of microbes often requires their cultivation, a problematic proposition as approximately only 1% of bacteria may be cultured by traditional methods. One promising strategy to grow the "unculturables" is to grow environmental microbes directly as mixed species biofilms – surface-bound, slime-encapsulated microbial communities. Mixed species biofilms can mimic natural environmental conditions and support microbial growth through beneficial social interactions between microbes, along with the inclusion of cell-cell signaling. Here, we describe a simple, flexible method for growing environmental mixed species biofilms in vitro using the Calgary Biofilm Device (CBD). Additionally, we describe a battery of assays for biofilm characterization. Using our approach, we have successfully grown mixed-species biofilms from a variety of hydrocarbon-contaminated environments, while demonstrating high retention of the original microbial diversity and the metabolic potential for biotechnology applications.

Keywords: Biofilms, Calgary Biofilm Device, Unculturables

1 Introduction

Microbes play an important role in the biogeochemistry of hydrocarbons [1, 2]. Capable of both synthesizing and degrading hydrocarbons, microbes impact environmental pollution (and therefore, by extension, human health) and are often harnessed for a range of hydrocarbon-focused biotechnology applications [3–6]. Thus, the study of microbes and their interactions with hydrocarbons is of great interest.

The empirical study of microbes often seeks their cultivation as individual isolates. But, molecular-based cultivation-independent investigations of microbial diversity and simple microscopic counts have both demonstrated that most microorganisms resist cultivation by traditional isolation methods [7, 8]. Some suggest that only

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approximately 1% of the bacteria on Earth have been isolated in vitro, while the remaining ~99% have been labeled "unculturable" [9]. It is important to understand that "unculturable" does not mean "will never be cultured" but rather that the current state of our knowledge and associated techniques of microbial growth is particularly lacking and falls short of being able to culture every environmental microbe as a single-species isolate at this time [10]. Fortunately, microbiologists have been developing new strategies for culturing "unculturable" bacteria. Such strategies include the use of dilute nutrient media (particularly for oligotrophs) and attempting to simulate natural environmental conditions [9].

Simulation of natural conditions includes abandonment of isolationist approaches, by promoting the simultaneous growth of the entire microbial community. Additionally, it has been suggested that bacteria in nature predominately exist as surface-bound, extracellular polymeric substance (EPS)-encapsulated, mixed-species consortia known as biofilms [11–13]. Sociality in microbial biofilms is highly influential; the close physical proximity of cells within a biofilm may lead to beneficial, social interactions including cross feeding or metabolic cooperation [14–17]. Another key social feature of biofilm communities is communication via signaling molecules (including quorum sensing) [18]. Empirical evidence suggests that cultivation strategies that include/offer beneficial bacterial interactions and opportunities for cell-cell signaling (such as growth in a biofilm) may be successful in co-culturing previously "unculturable" microbes [9, 10, 19]. Therefore, when attempting to study the microbial diversity, environmental stress tolerance, and metabolic potential of microorganisms from hydrocarbon-contaminated soils and waters as thoroughly as possible, our research group has adopted a multispecies biofilm approach.

Our approach to growing multispecies community biofilms utilizes a small-scale, high-throughput tool known as the Calgary Biofilm Device (CBD) [20]. Using this tool we have successfully cultured multispecies biofilms from the mature fines (sludge) of an Alberta oil sands tailings pond [21]. In support of the hypothesis that growth as a multispecies biofilm may cultivate the "unculturable" microbes, 16S rRNA gene 454 pyrosequencing analyses comparing the original sludge inoculum to that of the laboratory-grown biofilm community confirmed that our biofilm retained up to 75% of the original sludge OTUs [21]. We then expanded on this study by growing multispecies biofilms from the liquid fraction of an oil sands tailings pond and demonstrated that the intact biofilm community was more proficient at degrading naphthenic acids (oil sands tailings hydrocarbon contaminants) than biofilms of individual species isolated from tailings ponds using traditional plate methods [22].

In this chapter, we describe approaches for:

1. Cultivating an environmental mixed-species biofilm from both liquid- and soil/sediment-phase samples using the Calgary Biofilm Device (CBD)
2. Evaluating and characterizing the biofilms formed on the CBD using a battery of well-documented assays

2 Materials (*see* Note 1)

2.1 Cultivating an Environmental Mixed-Species Biofilm

2.1.1 Preparing Soil/Sediment-Phase Environmental Samples for CBD Inoculation

1. *Conical tubes*: 15 mL Falcon-like sterile, polystyrene centrifuge tube, Corning (VWR, https://ca.vwr.com/).

2.1.2 Inoculating CBD with an Environmental Sample

1. *Trypticase Soy Broth*: (VWR).
2. *Modified Bushnell-Haas minimal salts media (BH)*: 1 g KH_2PO_4, 1 g Na_2HPO_4, 0.5 g NH_4NO_3, 0.5 g $(NH_4)SO_4$, 0.2 g $MnSO_4•7H_2O$, 0.02 g $CaCl_2•2H_2O$, 0.002 g $FeCl_3$, 0.002 $MnSO_4•2H_2O$ per 1 L, pH 7, (analytical grade chemicals are used throughout) (*see* **Note 2**).
3. *Calgary Biofilm Device (CBD)*: MBEC-assays™ (Innovotech Inc, http://www.innovotech.ca/products_mbec.php) (Fig. 1).

2.2 Evaluation of Multispecies Biofilm Growth

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Plates*: Nunclon 96-well sterile microtiter plates (VWR).
3. *Recovery media*: 0.9% isotonic saline + 0.1% Tween 20 detergent (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).

2.2.1 Evaluating Biofilm Growth by General Cell Density

2.2.2 Evaluating Biofilm Growth by Dye Staining

Crystal Violet Assay

1. *Crystal violet dye*: crystal violet, Hardy Diagnostics (VWR international).
2. *Methanol*: 99.8% anhydrous methanol (Sigma-Aldrich).
3. *Plates*: Nunclon 96-well sterile microtiter plates (VWR).

Safranin Assay

1. *Safranin dye*: safranin, (VWR international).
2. *95% ethanol*: ethanol, 190 proof (Sigma-Aldrich).
3. *Plates*: Nunclon 96-well sterile microtiter plates (VWR).

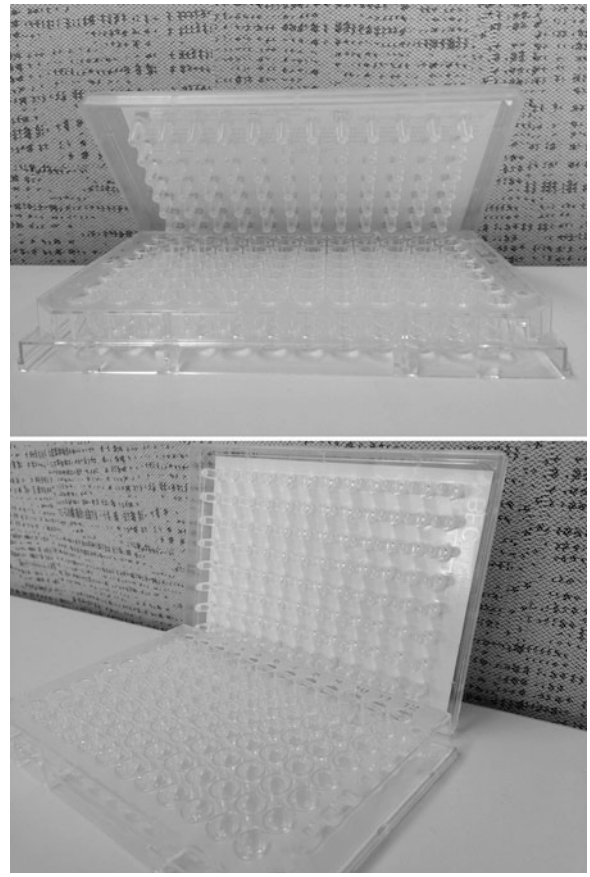

Fig. 1 The Calgary Biofilm Device (CBD), also known as an MBEC-assay™ (Innovotech Inc). The CBD is a two-part reaction vessel for growing biofilms in vitro consisting of a 96-well microtiter plate and a specialized lid with 96 identical polystyrene pegs on which biofilms attach and grow

2.2.3 Evaluating Biofilm Growth by Confocal Laser Scanning Microscopy

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Microscopy fixative*: electron microscopy grade, 70% glutaraldehyde (Cedarlane, https://www.cedarlanelabs.com/).
3. *Phosphate-buffered saline (PBS)*: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per 1 L, pH 7 (Sigma-Aldrich).
4. *Nucleic acid dye*: Syto® Red 62 nucleic acid stain (Invitrogen™| Life Technologies, http://www.lifetechnologies.com/ca/en/home/brands/invitrogen.html).

2.2.4 Semi-quantifying Biofilm Growth Using qPCR

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Genomic DNA Isolation Kit*: FastDNA® Spin Kit for Soil and FastDNA® Spin kit (MP Biomedical, http://www.mpbio.com/).

3. *Microfuge tubes*: 1.5 mL microcentrifuge tubes.
4. *Primers*: For 16S rRNA amplification; 926f (5°-AAACTYA AAKGAATTGRCGG-3°), and 1392r (5°-ACGGGCGGTGT GTRC-3°).
5. *SYBR Green Mix*: QuantiFast SYBR® Green PCR Kit (Qiagen, http://www.qiagen.com/products/catalog/assay-technologies/real-time-pcr-and-rt-pcr-reagents/quantifast-sybr-green-pcr-kit).
6. *Pseudomonas fluorescens Pf-5*: ATCC 13525.
7. *Lysogeny Broth (LB)*: or Luria Broth (VWR International).
8. *Quant-iT™ PicoGreen®*: Quant-iT™ PicoGreen® dsDNA assay kit (Life Technologies).
9. *Test tube*: Test tube, 10 mL, borosilicate glass.
10. *PCR tubes*: PCR Tubes and Caps, 0.2 mL.

2.3 Evaluation of Biofilm Cell Viability

2.3.1 Measuring ATP Levels as a Proxy for Biofilm Cell Viability

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Plates*: Nunclon 96-well sterile microtiter plates (VWR).
3. *Clear-bottom white plates*: Corning 96-well clear bottom white microplates (VWR).
4. *BacTiter-Glo reagent*: BacTiter-Glo™ Microbial Cell Viability Assay (Promega, https://www.promega.ca/products/cell-health-and-metabolism/cell-viability-assays/bactiter_glo-microbial-cell-viability-assay/).
5. *Minimal salts vitamin glucose (MSVG) media*: 1 g $(NH_4)SO_4$, 0.06 g $MgSO_4 \bullet 7H_2O$, 0.06 g $CaCl_2$, 0.02 g KH_2PO_4, 0.03 g $Na_2HPO_4 \bullet 7H_2O$, 2.383 g HEPES, 1 mL vitamins, 1 mL 10 mM $FeSO_4$, 0.998 g glucose per 1 L, pH 6.5. Reagents purchased from Sigma-Aldrich [23].
6. *Trace vitamins*: 20 mg biotin, 20 mg folic acid, 50 mg thiamine HCl, 50 mg D-(+)-calcium pantothenate, 1 mg vitamin B12, 50 mg riboflavin, 50 mg nicotinic acid, 100 mg pyridoxine HCl, 50 mg *p*-aminobenzoic acid per 1 L. Vitamins purchased from Sigma-Aldrich.

2.4 Evaluation of Multispecies Biofilm Composition

2.4.1 Fingerprinting Biofilm Bacterial Composition Using Denaturing Gradient Gel Electrophoresis

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Genomic DNA Isolation Kit*: FastDNA® Spin Kit for Soil (MP Biomedical).
3. *Microfuge tubes*: 1.5 mL microcentrifuge tubes.
4. *Primers*: For 16S rRNA amplification; 341f-GC (5′-CGCCCGC CGCGCCCCGCGCCCG TCCCGCCGCCCCCGCCCGCC TACGGGAGGCAGCAG-3′), and 907r (5′-CCGTCAATTCM TTTGAGTT-3′).

5. *Phusion® PCR Kit*: Phusion® High-Fidelity PCR Kit (New England BioLabs Inc, https://www.neb.com/products/e0553-phusion-high-fidelity-pcr-kit).
6. *PCR tubes*: PCR Tubes and Caps, 0.2 mL.
7. *50× TAE Buffer:* 2 M Tris Base, 1 M acetic acid, 50 mM EDTA volume to 1 L with ddH_2O, ph 8. Reagents purchased from Sigma-Aldrich.
8. *40% acrylamide/bis*: 38.93 g acrylamide, 1.07 g bis-acrylamide per 100 mL. Filter through a 0.45 micron filter and store at 4°C.
9. *0% denaturing stock with 6% acrylamide gel*: 15 mL 40% acrylamide/bis, 2 mL 50× TAE buffer, 83 mL ddH_2O. Filter through a 0.45 micron filter and store at 4°C in amber bottle for no more than 30 days. Reagents purchased from Sigma-Aldrich.
10. *100% denaturing stock with 6% acrylamide gel*: 15 mL 40% acrylamide/bis, 2 mL 50× TAE buffer, 40 mL formamide, 42 g urea, volume to 100 mL with ddH_2O. Filter through a 0.45 micron filter and store at 4°C in amber bottle for no more than 30 days (will require redissolving after storage). Reagents purchased from Sigma-Aldrich.
11. *30% denaturant*: 6 mL 100% denaturing stock, 14 mL 0% denaturing stock. For immediate use.
12. *60% denaturant*: 12 mL 100% denaturing stock, 8 mL 0% denaturing stock. For immediate use.
13. *10% ammonium persulfate (APS)*: 0.1 g ammonium persulfate per 1 mL (Sigma-Aldrich). Store at −20°C.
14. *N,N,N′,N′-Tetramethylethylenediamine (TEMED)*: *N,N,N′,N′*-Tetramethylethylenediamine, Sigma-Aldrich.
15. *SYBR® Gold*: 300 mL 1× TAE buffer, 30 µL SYBR® Gold Nucleic Acid Gel Stain (Life Technologies). Store at 4°C, may be used to stain a maximum of 5 gels.
16. *Stacking gel*: 625 µL 40% acrylamide/bis, 100 µL 50× TAE buffer, 4.275 mL ddH_2O, 4.5 µL TEMED, 20 µL APS. For immediate use.

2.4.2 Biofilm Bacterial Composition Using 16S rRNA Gene 454 Pyrosequencing

1. *0.9% isotonic saline*: 0.9 g of NaCl in 100 mL H_2O (or equivalent isotonic solution, depending on the nature of the environment) (*see* **Note 3**).
2. *Genomic DNA Isolation Kit*: FastDNA® Spin Kit for Soil (MP Biomedical).
3. *PCR tubes*: PCR tubes and caps, 0.2 mL (VWR International).
4. *Primers*: For 16S rRNA gene amplification; 926fw (5′-AAACT YAAAKGAATTGRCGG-3′), and 1392r (5′-ACGGGCGGTG TGTRC-3′).

5. *Primers*: For 16S rRNA gene with 454 adaptor and barcode sequences (XXX represents unique barcode sequence); 454T_RA_X (5′-CGTATCGCCTCCCTCGCGCCATCAGXXXXXXXXX[926fw]-3′), and 454T-FBw (5′-CTATGCGCCTTGCCAGCCCGCTCAGXXXXXXXXXX[1392r]-3′).
6. *Fermentas Master Mix*: Fermentas PCR Master Mix (2x) (Thermo Scientific, http://www.thermoscientificbio.com).
7. *QIAquick PCR Purification Kit*: QIAquick PCR Purification Kit, (Qiagen).

3 Methods

3.1 Cultivating an Environmental Mixed-Species Biofilm

Section 3.1 will highlight how to prepare your environmental sample for use as a biofilm inoculum source and how to grow environmental, mixed-species biofilms in a high-throughput, reproducible manner using the Calgary Biofilm Device (CBD), (MBEC™ assay, Innovotech) (*see* **Note 4**) (Fig. 2).

3.1.1 Preparing Soil/Sediment-Phase Environmental Samples for CBD Inoculation

In order to accurately and reproducibly inoculate the CBD, the environmental sample must reach a consistency that is distributable by micropipette. To achieve this, a soil/sediment slurry is created using ddH_2O at a ratio of 1:2.

1. Scoop ~5 mL of soil/sediment into a 15 mL conical tube.
2. Add 10 mL of ddH_2O (*see* **Note 3**).
3. Vortex (Scientiis Vortex Genie 2®) the mixture at the highest setting for approximately 1 min to obtain consistent slurry [21].

3.1.2 Inoculating CBD with an Environmental Sample

Culturing community biofilms turns out to be remarkably simple. The following protocol for growing community biofilms on the CBD mimics published procedures [20, 24–26].

1. Inoculate a multispecies community biofilm by pipetting a 1:1 mixture of the environmental sample (liquid or slurry (*see* Sect. 3.1.1)): twofold concentrated Tryptic Soy Broth (TSB) or Bushnell-Haas (BH) minimal salts media (*see* **Note 5**) to each well of the CBD to a final volume of 150 μL.
2. Place the inoculated CBD at 25–28°C on a gyro rotary shaker at 150 rpm under aerobic conditions (*see* **Notes 6** and **7**).
3. Incubate for a minimum of 2 days to allow for cellular adhesion to the CBD pegs and thus biofilm establishment (*see* **Notes 8** and **9**) [21, 22].

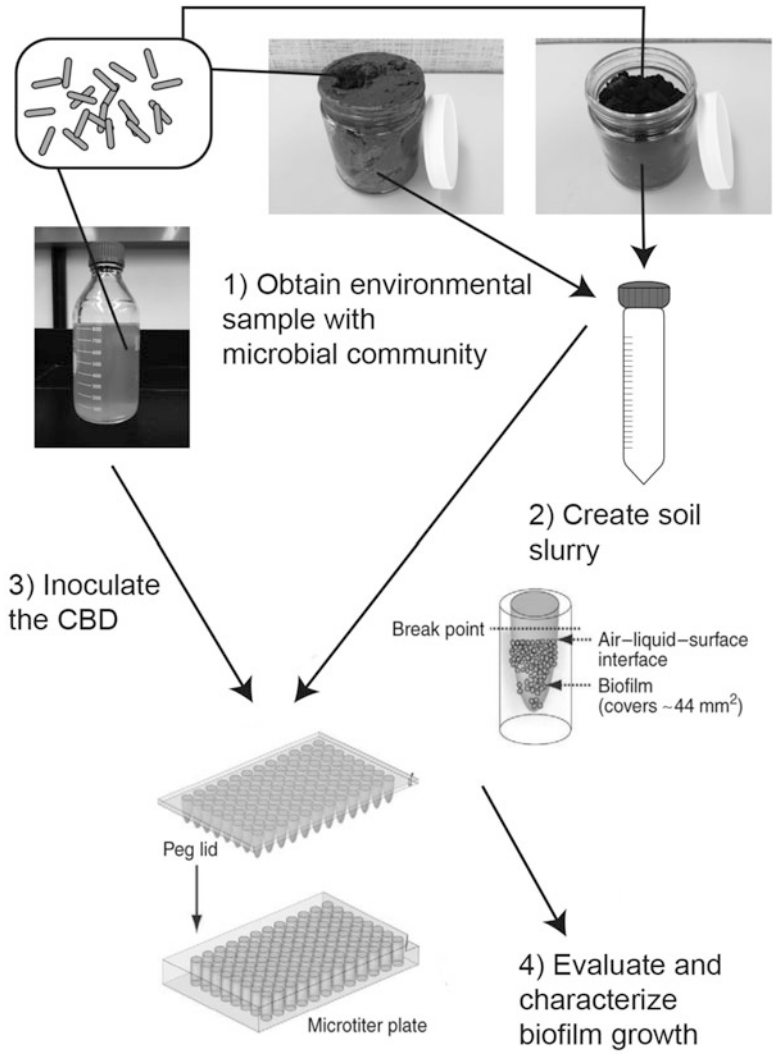

Fig. 2 Cultivating an environmental mixed-species biofilm using the Calgary Biofilm Device (CBD). (1) Both liquid- and soil/sediment-phase environmental samples may be used as an inoculum source. (*2*) Soil/sediment samples must first be diluted into slurry for ease of inoculation. (*3*) Samples are inoculated 1:1 with growth medium into the CBD and incubated. (*4*) After incubation, biofilm growth on CBD pegs is evaluated and characterized using a battery of documented methods. Note that individual pegs may be removed from the CBD lid at the ascribed breaking point for ease of manipulation and evaluation

3.2 Evaluation of Multispecies Biofilm Growth

The strength and appeal of the above procedure (Sect. 3.1) for growing community biofilms from environmental samples is due simply to the large number of assays available for evaluating and confirming biofilms grown in this manner.

3.2.1 Evaluating Biofilm Growth by General Cell Density

The optical density of intact cells dislodged from the CBD polystyrene pegs can be used as an approximation of the amount of biofilm growth [26].

1. Using microtiter plates, rinse the pegs of the CBD in 200 μL of ddH_2O twice to remove nonadherent biomass.
2. Remove the CBD lid from the rinse plate and place in a 96-well microtiter plate containing 200 μL of recovery media in each well.
3. Dislodge the biofilms from the pegs by sonication with a model 250T Sonicator (VWR International) for 30 min (use max power setting if applicable).
4. Discard the CBD lid and measure the optical density between 500 and 600 nm of the dislodged cells in the microtiter plate using a microtiter plate reader (Molecular Devices, Thermomax®) with data analysis software (Softmax Pro®).

3.2.2 Evaluating Biofilm Growth by Dye Staining

Bacteria and their associated extracellular polymeric substances (EPSs) that have adhered to the CBD polystyrene pegs may be simultaneously quantified using dye-staining methods. In this way, total biomass is evaluated.

Crystal Violet Assay

1. Using microtiter plates, rinse the pegs of the CBD in 200 μL of ddH_2O twice to remove nonadherent biomass.
2. Place the peg lid from the CBD into a 1% crystal violet solution for 10–15 min at room temperature.
3. Rinse the peg lid 3–4 times in 200 μL of ddH_2O to remove excess crystal violet.
4. Let the plate lid dry for 3 h or overnight.
5. Place the peg lid into 200 μL of 100% methanol in a 96-well plate for 15 min to solubilize the crystal violet stain.
6. Transfer 125 μL of the solubilized crystal violet solution into a new 96-well microtiter dish.
7. Measure the absorbance at 550 nm using a microtiter plate reader (PerkinElmer Enspire®) using 100% methanol as the blank, to quantify the biomass [26–28].

Safranin Assay

1. Using microtiter plates, rinse the pegs of the CBD in 200 μL of ddH_2O twice to remove nonadherent biomass.
2. The CBD pegs are then stained with 200 μL of a 0.1% safranin solution for 20 min at room temperature.
3. Rinse the peg lid 3–4 times in 200 μL of ddH_2O to remove excess safranin.
4. Place the peg lid into 200 μL of 95% ethanol in a 96-well plate for 5 min to solubilize the safranin stain.
5. Measure the absorbance at 550 nm using a microtiter plate reader (Molecular Devices Thermomax®) with data analysis software (Softmax Pro®) [29].

3.2.3 Evaluating Biofilm Growth by Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy is a useful tool for confirming the presence of a biofilm on the pegs of the CBD [21, 22, 25, 30–33]. Additionally, CLSM may be used to differentiate live cells from dead (*see* **Note 10**).

1. Rinse the planktonic cells from the CBD pegs using 200 µL of 0.9% saline.
2. Working in a fume hood, place the pegs in 200 µL of 5% glutaraldehyde (prepared in PBS) for 30 min to fix the biofilm. Fixation is necessary to preserve biofilm microstructure.
3. Stain the biofilms with 10 µM Syto® Red 62 nucleic acid stain for 15 min (*see* **Note 11**).
4. Visualize the biofilm via CLSM (Leica DM IRE2; Leica, USA) using a Texas Red Filter (excitation 550 nm; emission 600 nm) with a 64× water immersion objective (*see* **Note 12**).

3.2.4 Semi-quantifying Biofilm Growth Using qPCR

Once isolated from biofilm cultures, genomic DNA may be used to semi-quantify microbial growth by utilizing quantitative polymerase chain reaction (qPCR) to enumerate 16S rRNA transcripts (*see* **Note 13**) [21, 22]. Semi-quantification by qPCR requires the production of qPCR standards of known 16S rRNA gene copy number in order to create a standard curve in which to compare unknown sample 16S rRNA gene copy numbers. Since its genome has been annotated and therefore 16S rRNA gene copy numbers are known, we use pure cultures of the soil organism *Pseudomonas fluorescens* Pf-5 to create our qPCR standards.

qPCR Standards

1. Grow an overnight test tube culture of *P. fluorescens* Pf-5 (ATCC 13525) in 5 mL of LB broth at 25°C.
2. Place 1 mL of the *P. fluorescens* culture in a 1.5 mL microcentrifuge tube and centrifuge at 5,000 *g* for 10 min.
3. Discard supernatant and repeat until all *P. fluorescens* culture has been harvested.
4. Resuspend cells in 400 µL of isotonic saline.
5. Transfer 200 µL to (two) separate lysing matrix A tubes from an MP Bio FastDNA® Spin Kit.
6. Extract the genomic DNA using the bead-beating method of the FastDNA® Spin Kit as per the manufacturer's instructions.
7. Suspend isolated genomic DNA in 100 µL of the molecular grade H_2O provided in the FastDNA® Spin Kit. Combine both extracts in one, 1.5 mL microcentrifuge tube for a total volume of 200 µL.
8. Quantify the genomic DNA using a Quant-iT™ PicoGreen® dsDNA assay kit according to the manufacturer's instructions.

9. Knowing that *P. fluorescens* Pf-5 has a genome length of 7,074,893 bp and 5 copies of the 16S rRNA gene [34], use formula [1] to determine how many 16S rRNA gene copies exist per 2.5 μL of extract (qPCR reaction volume) [35].

$$\begin{aligned}\text{Gene copies} = (&\text{DNA concentration [g/μL]}) \\ &\times \left(\frac{1 \text{ mol bp DNA}}{660 \text{ g DNA}}\right) \\ &\times \left(\frac{6.023 \times 10^{23} \text{ bp}}{\text{mol bp}}\right) \\ &\times \left(\frac{\#\ 16\text{S rRNA copies}}{\text{genome size [bp]}}\right) \\ &\times 2.5\ \mu\text{L reaction volume} \end{aligned} \quad (1)$$

10. Generate the remaining qPCR standards by making tenfold serial dilutions of the DNA extract in molecular grade H_2O in 1.5 mL microcentrifuge tubes, stopping at 10^2 copies per qPCR reaction (*see* **Note 14**).

16S rRNA Gene Quantification by qPCR

1. Following the incubation period, rinse the peg lid of the CBD, twice, in 200 μL of 0.9% saline for 1 min.
2. Remove 2 identical pegs per sample – aseptically with sterilized pliers – into the Lysing Matrix E tube from a FastDNA® Spin Kit for Soil from MP Bio.
3. Subject the biofilm-containing pegs to a bead-beating extraction method with a FastDNA® Spin Kit for Soil following the manufacturer's instructions.
4. Suspend the isolated genomic DNA from the biofilm samples in 100 μL of molecular grade H_2O. This can be stored at −20°C until needed.
5. Make a qPCR master mix (per sample) consisting of 0.5 μL each of the bacterial 16S rRNA primers [forward = 926f (20 pmol/μL), reverse = 1392r (20 pmol/μL)], 9 μL molecular grade H_2O, and 12.5 μL of QuantiFast SYBR® Green super mix from Qiagen (http://www.qiagen.com).
6. Add 2.5 μL of genomic biofilm DNA to 22.5 μL of qPCR master mix.
7. Run the qPCR using a Rotor – Gene qPCR thermo cycler with the following cycle conditions: (1) 95°C for 5 min, (2) 95°C for 30 s, (3) 55°C for 45 s, and (4) 72°C for 90 s X 40 cycles.
8. Resolve the gene copy number per peg of the CBD for biofilm populations using formula [2] [21, 22].

$$\text{Gene copies per peg} = \frac{(\text{gene copies per reaction}) \times (100\ \mu\text{L DNA extract})}{(2.5\ \mu\text{L DNA per reaction}) \times (2\ \text{CBD pegs})} \quad (2)$$

3.3 Evaluation of Biofilm Cell Viability

3.3.1 Measuring ATP Levels as a Proxy for Biofilm Cell Viability

Measuring ATP levels in a cell culture is an effective way to follow the culture viability as only catabolically active cells produce ATP, and upon cell death ATP pools rapidly extinguish [36–38]. The strength of this ATP assay for cell viability is that it relies on luminescence and is plate-culture independent (*see* **Note 15**).

1. Following the incubation period, rinse the peg lid of the CBD, twice, in 200 μL of 0.9% saline for 1 min.
2. Place the CBD lid into a white-walled and clear-bottom 96-well microtiter plate containing 100 μL of MSVG growth media and 100 μL BacTiter-Glo reagent (BacTiter-Glo™ Microbial Cell Viability Assay, Promega) per well.
3. Incubate on a gyro rotary shaker (medium setting) at room temperature for 8 min.
4. Use a plate luminometer (PerkinElmer 2030 Victor X4) to measure assay luminescence (*see* **Note 16**).

3.4 Evaluation of Multispecies Biofilm Composition

3.4.1 Fingerprinting Biofilm Bacterial Composition Using Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a suitable, inexpensive technique to fingerprint and characterize microbial communities from natural ecosystems [39–41]. But, DGGE is a low-resolution technique, only capable of detecting the top 1% of the microbes present in a bacterial community [42]. Nonetheless, it is a good technique for elucidating large-scale community structure changes between samples [43]. The following protocol assumes the use of a Protean® 1-D Electrophoresis Chamber (Bio-rad, http://www.bio-rad.com/en-ca/category/large-format-1-d-electrophoresis-chambers?pcp_loc=catprod).

1. Following the incubation period, rinse the peg lid of the CBD, twice, in 200 μL of 0.9% saline for 1 min.
2. Remove 2 identical pegs per sample – aseptically with sterilized pliers – into the Lysing Matrix E tube from a FastDNA® Spin Kit for Soil from MP Bio.
3. Subject the biofilm-containing pegs to a bead-beating extraction method with a FastDNA® Spin Kit for Soil following the manufacturer's instructions.
4. Suspend the isolated genomic DNA from the biofilm samples in 100 μL of molecular-grade H_2O. This can be stored at −20°C until needed.
5. Using the contents of the Phusion® PCR Kit, make a PCR master mix (per sample) consisting of 2.5 μL each of the 16S rRNA

primers [forward = 341f-GC (10 pmol/μL), reverse = 907r (10 pmol/μL)], 10 μL HF buffer, 1 μL DNTP's, 1.5 μL DMSO, 2.5 μL $MgCl_2$ (10 mM) 28.5 μL molecular-grade H_2O, and 0.5 μL Phusion® high-fidelity polymerase.

6. Add 1 μL of genomic biofilm DNA to 49 μL of PCR master mix.
7. Run the PCR using a T100™ (Bio-Rad) thermal cycler with the following cycle conditions: (1) 98°C for 3 min, (2) 98°C for 10 s, (3) 52°C for 30 s, and (4) 72°C for 45 s X 30 cycles.
8. *Gel casting*: Two clean glass slabs are assembled into the Protean® gel-casting apparatus, as per the manufacturer's instructions, and tested for leaks with ddH_2O; remove residual H_2O.
9. Prepare 0% and 100% denaturant stocks with 6% acrylamide.
10. Prepare 20 mL of the low (30% denaturant) and 20 mL of the high (60% denaturant) gradient solutions from 0% and 100% denaturant stocks (*see* **Note 17**).
11. Add 10 μL TEMED and 100 μL APS (10%) to each low- and high-denaturant gradient solutions (these are catalysts and oxidants respectively to help crosslink the acrylamide – work quickly).
12. Pour the low- and high-gradient solutions into the appropriate chambers of the gradient maker (Jule Gradient Former™), and use the delivery tube to slowly and consistently (stir constantly) pour the gradient gel between the casting glass slabs.
13. To prevent gel desiccation during polymerization, cap gel with 1 mL of water-saturated 1-butanol.
14. Allow denaturing gel to polymerize for 1.5–2 h.
15. While gel polymerizes, setup Protean® electrophoresis apparatus with 1× TAE buffer at 60°C.
16. Once polymerized, drain 1-butanol and add stacking gel, insert well-forming comb, and allow 30 min for stacking gel polymerization.
17. Add 12.5 μL 5× DGGE loading dye into each 50 μL PCR product sample.
18. Load 50 μL of each PCR product sample into separate wells.
19. Run the gel at 60°C and 60 V for 19 h.
20. Disassemble and drain the Protean® apparatus.
21. Lay glass slab sandwich down on paper towel and carefully pry apart.
22. Carefully remove stacking gel.

23. Gently remove the gel and stain with SYBR™ Gold for 30 min.
24. Visualize on trans-illuminator with a SYBR™ Gold filter [21, 22].
25. Visual observation of DGGE band profiles allows for comparisons of biofilm microbial diversity between biofilm/sample types [21, 22].

3.4.2 Biofilm Bacterial Composition Using 16S rRNA Gene 454 Pyrosequencing

A more comprehensive, large-scale genotyping of the biofilm community is possible with next-generation high-throughput sequencing [44, 45]. Indeed, pyrosequencing has been used with great success to elucidate the diversity and composition of microbial biofilm communities, at resolutions far greater than those achieved by methods such as DGGE [21, 46–48]. The following procedure outlines our method for pyrosequencing of 16S rRNA gene transcripts:

1. Following the incubation period, rinse the peg lid of the CBD, twice, in 200 μL of 0.9% saline for 1 min.
2. Remove 2 identical pegs per sample – aseptically with sterilized pliers – into the Lysing Matrix E tube from a FastDNA® Spin Kit for Soil from MP Bio.
3. Subject the biofilm-containing pegs to a bead-beating extraction method with a FastDNA® Spin Kit for Soil following the manufacturer's instructions.
4. Suspend the isolated genomic DNA from the biofilm samples in 100 μL of molecular-grade H_2O. This can be stored at −20°C until needed.
5. For each sample (*see* **Note 18**) amplify the 16S rRNA gene(s) from the raw DNA using the following PCR protocol:
   a. Per 50 μL reaction: 2 μL DNA, 25 μL Master Mix (Fermentas), 21 μL molecular-grade H_2O, 1 μL of each primer [forward = 926fw (20 pmol/μL), reverse = 1392r (20 pmol/μL)].
   b. Cycle conditions: (1) 95°C for 30 s, (2) 55°C for 45 s, and (3) 72°C for 90 s, x 25 cycles.
6. The PCR product is then subjected to another round of PCR in order to generate a product with adaptor and bar coding sequences required for 454 pyrosequencing [21, 46].
   a. Per 50 μL reaction: 2 μL DNA, 25 μL Master Mix (Fermentas), 21 μL molecular-grade H_2O, 1 μL of each primer [454T_RA_X (20 pmol/μL), 454T-FBw (20 pmol/μL)].
   b. Cycle conditions: (1) 96°C for 45 s, (2) 65°C for 30 s, and (3) 72°C for 90 s, x 10 cycles.

7. Purify second PCR product with a QIAquick PCR purification kit.
8. Outsource pyrosequencing of samples (5–7 ng/μL) to Genome Quebec (McGill University Innovation Centre, Montreal, Canada) for sequencing with a Genome Sequencer FLX instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation) (*see* **Note 19**).
9. Process pyrosequencing 16S rRNA gene sequence data using the script developed by the Sun Center of Excellence for Visual Genomics (Calgary, Canada) (*see* **Note 20**). Subject sequencing data to stringent quality controls; sequences are eliminated if adaptor and primer sequences do not match perfectly, ambiguous bases were introduced, sequence quality score falls below 25, and if homopolymer lengths are greater than 8 bp. Further screen high-quality sequences for problematic, chimeric, and eukaryotic sequences using the Tera-BLAST algorithm on the TimeLogic Decypher system (Active Motif Inc.). Sequences are excluded if they have a BLAST e-value greater than e-50 or if their best alignment coverage is less than 80%. Filtered sequences are then clustered into OTUs at 3% distance using the linkage algorithm in Mothur. Taxonomic consensus from each OTU is then achieved with a BLAST search against the SILVA database. Good reads are assigned at the phylum, class, and/or genus level [21] (*see* **Note 21**).

4 Notes

1. In the Materials section, we list the vendors (along with vendor URLs) that we purchased our reagents and consumables from for the benefit of the reader. These are in no way endorsements of these vendors or their products, and we encourage readers to utilize suitable replacements at their discretion.
2. Alternative media can be used to suite the environment. Bushnell-Haas media has proven successful for culturing microbes as biofilms from hydrocarbon-contaminated environments.
3. Please consider the salinity of the environment from which your inoculant is sourced, and adjust diluents and rinse solutions as necessary. For example, salinity greater than 0.9% may be necessary to maintain isotonic conditions when working with marine (~3.5% saline) sediments.
4. The Calgary Biofilm Device (CBD) is a 96-well microtiter plate-based 2-part reaction vessel for growing reproducible biofilms. Its high-throughput design allows for the study of multiple parameters (toxicants, growth media, etc.) at one time [20]. We recognize that biofilms may be grown on the walls of a

standard microtiter plate; however, the use of the CBD is more advantageous for the following reasons: (1) Biofilms grown in the CBD form on the specialized polystyrene pegs, which provide an additional 109 mm^2 of surface area for cell attachment as compared to standard microtiter plates [25]. (2) CBD pegs are removable from the lid, which allows for many more downstream assessments of biofilm growth than are available for biofilms grown in standard microtiter plates.

5. The Bushnell-Haas minimal salts media was amended with various carbon sources at different concentrations. The amendments include no carbon source addition, 0.001–1 g/L of molasses (from the local grocer), peptone (VWR), yeast extract (VWR), and glucose (VWR).
6. The chosen RPM is relatively arbitrary; the goal is simply to provide sheer force to stimulate cell attachment to the peg surface. We have experienced positive results at both 125 and 150 rpm and therefore suggest that RPM may be adjusted at your discretion.
7. Depending on the oxygen tension of your environmental sample, anaerobic or microaerophilic conditions may be required to grow a biofilm. Using the procedure listed in Sect. 3.1, we have successfully grown anaerobic biofilms from an oil sands mature fine tailings sample using atmospheric conditions of 90% N_2 and 10% CO_2/H_2, as well as microaerophilic biofilms in candle jars [21].
8. Different growth periods may be required depending on the microorganisms being grown. Throughout the incubation period, it may be beneficial to provide the biofilms with a fresh supply of growth media; to do this, simply prepare a new microtiter plate with 150 μL of fresh growth media per well, and aseptically transfer the CBD lid from the old plate to the fresh plate.
9. The primary version of the CBD contains polystyrene pegs, of which the chemical properties of polystyrene may or may not be suitable for the adhesion process for the types of microbes in the community of interest. For example, adding an electrostatic charge to the polystyrene may serve to help cells attach. We have found that simply soaking the pegs (placing CBD lid in 96-well microtiter plate containing a solution of the compound to be coated onto the CBD pegs) in various solutions will provide a "conditioning film" on the peg. This allows one to change the charge (using lysine, aspartate, alanine, succinate, acetate) or degree of hydrophobicity (using glycerol, polyethylene glycol) to tailor and adjust the chemical properties of the peg surface [49]. The high-throughput nature of microtiter plates makes finding the appropriate peg coating relatively

easy. Potential coatings can be screened by diluting a particular compound through a column in a microtiter plate, allowing 12 different compounds/coatings to be tested at different concentrations. The CBD lid is soaked in this coating screening plate and subsequently inoculated. Assessment of biofilm growth on all pegs should reveal which coating (and at what particular concentration) results in the best biofilm growth.

10. A Live/dead® BacLight™ bacterial viability kit (Invitrogen), which employs the two stains Syto-9 and propidium iodide, is an alternative to the use of Syto® red 62 and differentiates between live cells (green) and dead cells (red) [25, 50]. If live/dead staining is to be performed, glutaraldehyde fixation is avoided to prevent undesired cell death.
11. An alternative stain is Acridine Orange (Sigma Aldrich), prepared at 0.1% in PBS (Sect. 2.2.3). Stain the CBD pegs in 0.1% Acridine Orange for 5 min. Note that hydrocarbon autofluorescence may interfere with the emission fluorescence of Acridine Orange [21]. Fluorophore-conjugated lectins have been used for staining biofilm extracellular polysaccharides [25].
12. CBD pegs may be removed with flame-sterilized pliers (from your local hardware store) for ease of manipulation on the microscope.
13. Counting the number of 16S rRNA genes in a biofilm is only semiquantitative and represents an estimation of the bacterial numbers, as bacteria often possess more than one copy of the gene [51, 52].
14. Our *P. fluorescens*-derived qPCR standard curve ranges from 9×10^7 to 9×10^2 copies/rxn.
15. More traditional biofilm cell viability assays employ a colony-forming unit (CFU) method in which biofilm cells must be cultured on agar plates [20, 23, 26, 53]. Although it is possible to conduct CFU viability counts on environmental multispecies biofilms, this is undesirable due to the loss of any microbes not able to grow on the agar media ("unculturables").
16. Sample luminescence is normalized to background luminescence (measured from a mixture of 100 μL of MSVG medium and 100 μL of BacTiter-Glo reagent).
17. The denaturant gradient described herein resulted in adequate separation of 16S rRNA gene fragments from our biofilm communities but may not be applicable for all biofilm communities. Gradient optimization may be required.
18. Don't forget to include your environmental sample in your analysis to allow for comparisons between cultured multispecies biofilms and the native environmental community.

19. The availability of various second- and third-generation sequencing technologies and focused companies worldwide provide multiple options for this.
20. Two, online, more freely accessible alternative pipelines for processing of pyrosequencing data are the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [54] and the Phoenix 2 ribosomal RNA gene sequence analysis pipeline [55].
21. Once one has performed community profiling of the environment and grown the full community, one may wish to have more control over the culture. Through the bioinformatics, a representative species in a given clade can be identified that can be cultured as a single species. With a representative species of each clade identified, one can then establish mixed-species communities both planktonic and as a biofilm of this select subset to provide a model of the whole. We have done this for a water distribution system [50]. A key to success here is to realize that fast-growing species can quickly overwhelm the community. Additionally, many organisms when grown individually require special media conditions. The first step is to identify a media formulation that allows all isolates to grow to some extent. With this media in hand, a growth curve is performed. Then an empirically determined inoculation strategy must be formulated that begins with the slower growers inoculated first, following those of increasing growth rates. Once the community is growing, a natural population balance will form. In regard to biofilms, a biofilm attachment experiment is necessary. Different bacteria will adhere to the surface chosen for the model system to differing extents. Some community biofilms require some organisms to colonize the sterile surface first, then followed by others adding into the sessile community.

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Cultivation of Hydrocarbon-Degrading Fungi

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Abstract

Fungi form the biggest microbial biomass and are key drivers of hydrocarbon biodegradation. We here describe standard cultivation methods for fungi that use hydrocarbons either as sole carbon and energy source or co-metabolically degrade them. The methods include both liquid (Protocols 1 and 2) and solid-state cultivation approaches (Protocol 3) as well as a versatile protocol to monitor fungal biomass based on the quantification of the specific fungal marker ergosterol (Protocol 4).

Keywords: Co-metabolism, Enzymes, Ergosterol, Fungi, Liquid cultivation, Solid-state cultivation

1 Introduction

The kingdom "Fungi" includes moulds, mushrooms, lichens, rusts, smuts and yeasts and currently contains 79,000 to 100,000 described species [1]. Many fungi possess the biochemical and ecological capacity to degrade hydrocarbons and to decrease their risk to humans and the environment [2]. In general, these fungi have two mechanisms to act on hydrocarbons: (1) utilisation of hydrocarbons as a source for carbon and energy and (2) co-metabolic degradation in the presence of a second substrate serving as carbon and energy source [2]. A peculiarity is their ability to degrade organic compounds by extracellular oxidoreductases of highly unspecific activity. Such enzymes have evolved to support fungal growth on complex, recalcitrant lignocellulosic substrates via the depolymerisation and removal of lignin and its humic substance derivatives [2]. Furthermore, intracellular hydrocarbon-oxidising enzymes such as cytochrome P-450 monooxygenase systems contribute to the considerable unspecificity of fungal attack on hydrocarbons. This is in contrast to bacterial hydrocarbon degradation where bacteria typically employ specific biochemical pathways to use contaminants as growth substrates [2]. Bacterial growth on hydrophobic compounds such as hydrocarbons is hence often challenged by a limited substrate availability, i.e. sufficient substrate delivery to the microorganisms [3]. Hydrocarbons

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such as PAH are typically degraded co-metabolically by fungi belonging to the phyla Ascomycota and Basidiomycota. Filamentous wood- and soil-litter-decaying basidiomycetes frequently use unspecific oxidative exoenzymes employed in lignin and lignocellulose decomposition to mineralise hydrocarbons co-metabolically, i.e. in the presence of an additional lignocellulosic substrate serving as carbon and energy source. Other hydrocarbons such as certain BTEX constituents (e.g. toluene, ethylbenzene) or *n*-alkanes are utilised by a range of hydrocarbon-degrading fungi as sole sources of carbon and energy [2].

Hydrocarbon-degrading fungi can be cultivated in both liquid- and solid-state cultivation approaches enabling growth either by degradation of hydrocarbons as sole carbon and energy source (Protocol 1) or by co-metabolic degradation of hydrocarbons while using synthetic or complex natural carbon sources as growth substrate (Protocols 2 and 3). Fungi already known to degrade hydrocarbons may be derived from public culture collections, e.g. the strain collection of the Centraalbureau voor Schimmelcultures (CBS) or the American Type Culture Collection (ATCC). In order to obtain co-metabolic hydrocarbon degraders from environmental samples (e.g. soil, water, plant material, etc.), fungi first may be isolated using common agar plate-based isolation techniques and subsequently tested for degradation of the pollutant hydrocarbon of concern [4, 5]. Fungi capable of utilising hydrocarbons as sole source of carbon and energy can be enriched from environmental samples prior to their isolation. This can be achieved by supplementation with the hydrocarbon to be degraded as the only carbon and energy source, thereby providing growth conditions selectively favouring fungi with the desired properties. For instance, fungi growing on toluene were enriched prior to their isolation in a compost biofilter used to remove toluene from contaminated air [6]. Generally, antibacterial antibiotics are frequently applied during the isolation of fungi to suppress contaminating bacteria [4, 6]. Furthermore, media and further conditions applied for the isolation and cultivation of fungi may be designed to mimic the natural habitat conditions with respect to, e.g. pH, temperature and salinity [5, 7, 8]. Table 1 gives a structured overview on the cultivation approaches and details their frequent applications and challenges in research- and technology-based uses. For instance, biomass quantification and determination of fungal growth rates by optical density measurements are only possible for fungi growing as single cells (e.g. yeasts). As filamentous fungi tend to form aggregates (pellets) in shaken liquid cultures and frequently grow in the form of dense mycelial mats at the surface of non-agitated liquid cultures, more laborious approaches such as the determination of the weight of the dry fungal biomass, the quantification of ergosterol or certain phospholipid fatty acids as specific markers of fungal biomass [9] or quantitative molecular biological (e.g. qPCR based)

Table 1
Overview of liquid- and solid-state cultivation approaches for fungal growth based on the utilisation of hydrocarbons (=HC) for growth and their co-metabolism

	Growth on hydrocarbon: degradation of HC as sole carbon and energy source	Co-metabolic degradation of hydrocarbon: growth on secondary carbon sources
Liquid cultures	**Frequent use** – Elucidation of metabolic pathways of HC degradation, parent compound degradation and metabolite formation kinetics and rate-limiting steps – Assessment of toxicity thresholds of HC and their metabolites – Elucidation of environmental boundary conditions for HC degradation – Selective enrichment of HC-degrading fungal strains from environmental sources	**Frequent use** – Elucidation of metabolic pathways of HC degradation, parent compound degradation and metabolite formation kinetics and rate-limiting steps – Assessment of toxicity thresholds of HC and their metabolites – Elucidation of environmental boundary conditions for HC degradation
	Remarks – Growth in liquid media on dissolved HC or HC present in a second phase – Limited HC bioavailability can reduce fungal growth rate – Toxicity of HC can limit fungal growth – Fungal growth can be easily determined by standard protocols – Biomass quantification by optical density only possible for fungi growing as single cells (e.g. yeasts) → ***Protocol 1***	**Remarks** – Growth on dissolved secondary carbon sources (i.e. synthetic or complex natural) in the presence of dissolved HC or HC present in a second phase – Limited HC bioavailability can limit fungal HC degradation – Toxicity of HC can limit fungal growth – Fungal growth can be easily determined by standard protocols – Biomass quantification by optical density only possible for fungi growing as single cells → ***Protocol 2***
Solid-state cultivation	**Frequent use** – Often used in practical applications for clean-up of waste air streams contaminated with volatile HC pollutants – Used to enrich fungi capable of growing on volatile HC pollutants	**Frequent use** – Assessment of HC degradation potential of fungi – Cultivation of fungi at environmentally relevant conditions
	Remarks – Growth on a solid, poorly degradable support matrix in the presence of gaseous HC or HC present in a second phase – Toxicity of HC can limit fungal growth rates – Provision of sufficient humidity is required for optimal fungal growth – Assessment of fungal growth is more laborious due to difficult separation of	**Remarks** – Growth on a solid, complex carbon source as support matrix in presence of dissolved HC or HC present in a second phase – Limited HC bioavailability can limit fungal HC degradation rates – Assessment of fungal growth is more laborious due to difficult separation of biomass components from the matrix;

(continued)

Table 1
(continued)

	Growth on hydrocarbon: degradation of HC as sole carbon and energy source	Co-metabolic degradation of hydrocarbon: growth on secondary carbon sources
	biomass components from the support matrix – Less suitable method for the elucidation of metabolic pathways of HC degradation and metabolite formation kinetics	biomass quantification, e.g. by ergosterol extraction and analysis – Application of isotopically labelled HC substrates (e.g. ^{14}C or ^{13}C) often used for the quantification of degradation rates and metabolite assessment – Heterogeneity of the system may reduce possible toxic effects of HC → **Protocol 3** → **Protocol 4**

methods need to be applied. In solid-state cultivation, the monitoring of fungal biomass may become even more complex due to difficult separation of biomass or its markers from the solid support matrix. Hence, in addition to the three cultivation procedures, this chapter describes a versatile protocol for the quantitative extraction and determination of ergosterol from complex solid growth substrates (Protocol 4).

2 Materials

If not stated otherwise, all reagents can be purchased, e.g. from Sigma-Aldrich (http://www.sigmaaldrich.com).

2.1 Growth of Fungi on Hydrocarbon Pollutants in Liquid Culture (Protocol 1)

1. *Fungal strain*: *Cladosporium sphaerospermum* CBS 114326 (Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, an institute of the Royal Netherlands Academy of Arts and Sciences (KNAW), CBS strains collection (http://www.cbs.knaw.nl/Collections); other collections: ATCC 200384, American Type Culture Collection, ATCC (http://www.lgcstandards-atcc.org/)) (*see* **Note** 1).
2. Malt extract peptone agar plates for strain maintenance at 28°C: 3% (w/v) malt extract, 0.3% (w/v) soya peptone, 1.5% (w/v) agar (pH 5.6).
3. Mineral medium according to Hartmans et al. [10], containing per L: 0.85 g $Na_2HPO_4 \cdot 2\ H_2O$, 1.55 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 0.20 g $MgSO_4 \cdot 7\ H_2O$, 0.001 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $MgCl_2 \cdot 6\ H_2O$, 0.01 g EDTA, 0.005 g $FeSO_4 \cdot 7\ H_2O$, 0.002 g $ZnSO_4 \cdot 7\ H_2O$, 0.001 g $MnCl_2 \cdot 2\ H_2O$, 0.2 mg $NaMoO_4 \cdot 2\ H_2O$, 0.2 mg $CuSO_4 \cdot 5\ H_2O$, 0.4 mg

$CoCl_2 \cdot 6\ H_2O$ (for preparing agar plates, the medium has to be solidified with 15 g L^{-1} agar).

4. Toluene, anhydrous, 99.8%.
5. Sodium azide, purum p.a., ≥99.0%.
6. Ultra-Turrax® homogeniser for volumes from 0.5 to 100 mL (H_2O) (IKA, Staufen, Germany (http://www.ika.de)).

2.2 Fungal Cultivation for Co-metabolic Biotransformation of Pollutant Hydrocarbons in Liquid Culture (Protocol 2)

1. Fungal strain: *Clavariopsis aquatica* de Wild. strain WD(A)-00-1 (strain collection of the Department of Environmental Microbiology, Helmholtz Centre for Environmental Research – UFZ (http://www.ufz.de/index.php?en=13354)).
2. Malt extract agar plates for strain maintenance at 14°C: 1% (w/v) malt extract, 1.5% (w/v) agar (pH 5.6–5.8).
3. Liquid malt extract medium for liquid cultivation: 1% (w/v) malt extract (pH 5.6–5.8).
4. Nonylphenol, technical grade, mixture of ring and chain isomers.
5. Methanol, anhydrous, 99.8%.
6. Tween 80, viscous liquid.
7. Sodium azide, purum p.a., ≥99.0%.
8. Ultra-Turrax® homogeniser for volumes from 0.5 to 100 mL (H_2O) (IKA).

2.3 Fungal Solid-State Cultivation for Co-metabolic Biotransformation of Pollutant Hydrocarbons (Protocol 3)

1. Fungal strain: *Trametes versicolor* DSM 11269 (Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de)).
2. Malt extract agar plates for strain maintenance at 24°C: 2% (w/v) malt extract, 1.5% (w/v) agar (pH 5.6–5.8).
3. Milled wheat straw, <1 mm particle size (*see* **Note 2**).
4. [ring-U^{14}C]Phenanthrene, specific activity 50–60 mCi/mmol (1.85–2.22 GBq/mmol), concentration 0.1 mCi/mL in ethanol (BIOTREND Chemikalien GmbH, Köln, Germany (http://www.biotrend.com)).
5. Phenanthrene, 98%.
6. [4,5,9,10-^{14}C]Pyrene, specific activity 50–60 mCi/mmol (1.85–2.22 GBq/mmol), concentration 0.1 mCi/mL in ethanol (BIOTREND Chemikalien GmbH).
7. Pyrene, 98%.
8. Ethanol, ≥99.8%.

2.4 Extraction and Determination of Ergosterol as a Marker of Fungal Biomass (Protocol 4)

1. Ergosterol, ≥95.0% (HPLC).
2. Phenanthrene, 98%.
3. Toluene, anhydrous, 99.8%.
4. Methanol, anhydrous, 99.8%.
5. Methanol, gradient grade (Th. Geyer, Renningen, Germany (http://www.thgeyer.com)).
6. Ethanol, ≥99.8%.
7. Deionised water, e.g. prepared by Q-Gard 2 (Merck Millipore (http://www.merckmillipore.com)).
8. Filter paper, e.g. Whatman® Filter No. 1 (GE Healthcare Life Sciences, (http://www.gelifesciences.com)).
9. 15-mL and 50-mL Falcon® tubes (VWR International (http://vwr.com)).
10. Drying oven, e.g. ED 53 (BINDER, Tuttlingen, Germany (http://www.binder-world.com)).
11. Ball mill, e.g. Pulverisette 23 (Fritsch, Idar-Oberstein, Germany (http://www.fritsch-milling.com)).
12. Horizontal shaker, e.g. GFL model 3018 (GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany (http://www.gfl.de)).
13. Ultra Performance Liquid Chromatography (UPLC) system, e.g. Acquity™ UPLC System composed of a Binary Solvent Manager (BSM), a SampleManager (SM) and a PDA eλ photo diode array detector; equipped with an Acquity™ UPLC BEH C18 column (1.7 μm particle size; 2.1 × 50 mm) (Waters, Eschborn, Germany (http://www.waters.com)).

3 Methods

3.1 Growth of Fungi on Hydrocarbon Pollutants in Liquid Culture (Protocol 1)

The following protocol makes use of the fungus *C. sphaerospermum* CBS 114326 (ATCC 200384) growing on toluene to exemplify fungal growth on volatile aromatic hydrocarbons in liquid culture and has been adapted from Weber et al. [6], Herklotz [11] and Vogt et al. [12]. The approach may be modified to meet the requirements of also other fungi growing on similar pollutants. Sterile conditions have to be ensured during all of the following steps.

1. Use one agar plug (7 mm diameter) derived from the margin of well-grown fungal colonies maintained on malt extract peptone agar plates (*see* Subheading 2.1) at 28°C to inoculate a fresh agar plate containing mineral medium according to Hartmans et al. [10] (*see* **Note** 3 and Subheading 2.1). Place the inoculated agar plates in a desiccator, together with an open glass petri dish containing 15 mL pure toluene (*see* **Note** 3) to produce a toluene vapour and a beaker containing sterile 0.9%

NaCl solution to humidify the air in the desiccator. Incubate at ambient temperature until sufficient fungal growth is observed, which may take about 1–2 weeks (*see* **Note** 4).

2. Add 116 μL cooled toluene to a 2-L bottle containing 900 mL of sterile mineral medium according to Hartmans et al. [10], seal the bottle gastight with a crimped Teflon-coated butyl rubber stopper and shake overnight for equilibration [12]. The thus obtained toluene-spiked medium serves as a basis for liquid cultivation of *C. sphaerospermum* on toluene (*see* **Note** 4).
3. In order to prepare a liquid inoculum for subsequent degradation experiments, inoculate 120-mL serum bottles containing 50 mL of the toluene-spiked mineral medium described above with 6 fungus-overgrown agar plugs (7 mm diameter) derived from fungal cultivation on toluene-amended agar plates as described above. Homogenise the agar plugs with the help of using an Ultra-Turrax® (*see* Subheading 2.1), seal the serum bottles with crimped Teflon-coated butyl rubber stoppers and incubate at 28°C and 120 rpm until sufficient fungal growth is achieved (usually within 1–2 weeks, *see* **Note** 4).
4. For degradation experiments, inoculate 2-L flasks containing 900 mL of toluene-amended mineral medium with 100 mL of a liquid fungal pre-culture grown on toluene as described above. Uninoculated bottles where the fungal inoculum has been replaced by sterile water or inoculated bottles inactivated with sodium azide at 500 mg L^{-1} may serve as controls. Seal the bottles with crimped Teflon-coated butyl rubber stoppers, and incubate at 28°C and 120 rpm for about 10–12 days (*see* **Note** 4). Aliquots of the culture medium are to be taken regularly (starting from the time point of inoculation) by syringes for analysis of toluene (2 × 0.5 mL is recommended). The removed volume has to be balanced by sterile air to avoid negative pressure inside the bottles. Headspace gas chromatography for toluene analysis can be applied as previously described [12].

3.2 Fungal Cultivation for Co-metabolic Biotransformation of Pollutant Hydrocarbons in Liquid Culture (Protocol 2)

This protocol aims at providing environmental pollutants to fungal liquid cultures for co-metabolic biotransformation, thereby serving as a basis for studying the kinetics of conversion and metabolite formation. Here, we use the aquatic hyphomycete *Clavariopsis aquatica* de Wild. strain WD(A)-00-1 and technical nonylphenol (t-NP. an environmentally relevant mixture of isomers of predominantly 4-nonylphenol with variously branched nonyl chains; *see* Subheading 2.2) [4] to exemplify the approach, which in principle may be adapted also to other fungi and various organic environmental pollutants with low molecular weights. A peculiarity of the protocol described below is that a combination of a detergent and an organic solvent (Tween 80 and methanol) is applied to ensure full aqueous solubility of the hydrophobic pollutant of concern, in order to avoid bioavailability limitations caused by the limited

aqueous solubility of the target pollutant and to enable direct analysis of aqueous samples (i.e. cell-free culture supernatants) by routine (U)HPLC methods without the need for preceding extraction of analytes. Sterile conditions have to be ensured during all of the following steps.

1. Prepare a mycelial suspension (inoculum) by homogenising a defined number of agar plugs (7 mm diameter) derived from the margin of well-grown fungal colonies maintained on agar plates (*see* Subheading 2.2) at 14°C together with an equal amount of sterile distilled water (e.g. ten agar plugs together with 10 mL distilled water), using an Ultra-Turrax® (*see* Subheading 2.2).
2. Inoculate Erlenmeyer flasks (100 mL) containing 30 mL of a liquid malt extract medium (*see* Subheading 2.2) with 0.5 mL of a mycelial suspension, and incubate with agitation (120 rpm) at 14°C in the dark (*see* **Note** 4).
3. Prepare an aseptic 25 mM stock solution of t-NP in methanol, containing 10% (w/v) Tween 80 (*see* **Note** 5).
4. On culture day 5, aseptically add t-NP to fungal cultures from the stock solution described above to yield a final concentration of 250 μM (corresponding to final concentrations 1% (v/v) methanol and 0.1% (w/v) Tween 80) (*see* **Note** 5). For preparing inactive control cultures, add sodium azide at 500 mg L^{-1} to fungal cultures on culture day 4, and continue incubation for one more day (until culture day 5). Then, add t-NP to controls as outlined above.
5. Incubate the t-NP-amended fungal cultures over the desired time, take 0.5-mL samples from cell-free culture supernatants at the time intervals needed, mix with 0.5 mL methanol and store them at −20°C until pollutant analysis by (U)HPLC (*see* **Note** 6).

3.3 Fungal Solid-State Cultivation for Co-metabolic Biotransformation of Pollutant Hydrocarbons (Protocol 3)

The following protocol aims at the assessment of co-metabolic biotransformations of environmental pollutants during fungal growth on solid lignocellulosic substrates. Here, we describe the cultivation of the white-rot basidiomycete *Trametes versicolor* DSM 11269 (*see* Subheading 2.3) on wheat straw for investigating the co-metabolic mineralisation of the polycyclic aromatic hydrocarbon (PAH) representatives phenanthrene and pyrene as an example, which was adapted from previous studies [13, 14]. Likewise, the protocol may be adapted to other wood- and litter-decaying fungi, different lignocellulosic substrates and various low-molecular-mass hydrocarbons. For the described approach, the application of ^{14}C-labelled pollutants is a prerequisite for monitoring their complete biodegradation to CO_2 (mineralisation) and is further recommended for the generation of ^{14}C mass balances involving parent

pollutants and metabolites/products thereof [15]. Sterile conditions have to be ensured during all of the following steps.

1. Add 5 g (dry weight) of milled wheat straw (*see* Subheading 2.3) to 300-mL Erlenmeyer flasks, wet with 10 mL distilled water, and autoclave at 121°C for 40 min.
2. Suspend 250 μL of ethanolic phenanthrene or pyrene solution in 10 mL of sterilised water to finally yield a total of 0.5 μCi corresponding to 5 mg of ^{14}C-labelled and unlabelled PAH. Add the resulting suspensions to the straw-containing Erlenmeyer flasks under sterile conditions.
3. Inoculate each Erlenmeyer flask with five agar plugs (7 mm diameter) derived from the margin of well-grown fungal colonies maintained on malt extract agar plates at 24°C (*see* **Note** 4 and Subheading 2.3). Leave a desired amount of flasks uninoculated as controls.
4. Seal flasks gastight with crimped butyl rubber stoppers and incubate them at 24°C without agitation over the desired time period (e.g. 40–60 days) (*see* **Note** 4). During cultivation, aerate flasks continuously or at suitable regular intervals, trap $^{14}CO_2$ using either NaOH or a CO_2-binding liquid scintillation fluid and quantify the trapped $^{14}CO_2$ by liquid scintillation counting according to previously described protocols [13, 14].
5. If desired, harvest flasks at the end of the experiment, and generate ^{14}C mass balances involving different parent pollutant- and metabolite-containing fractions according to previously established methods [15] (*see* **Note** 7).

3.4 Extraction and Quantification of Ergosterol as a Marker of Fungal Biomass (Protocol 4)

The following protocol aims at the extraction and quantification of the fungal cell membrane component ergosterol from fungi-containing solid substrates (e.g. lignocellulosic materials such as straw, husk or soil), in order to enable an estimate of fungal biomass under conditions where fungi cannot quantitatively be separated from their substrates/the environmental matrix they are inhabiting. Based on the recorded ergosterol contents, the corresponding fungal biomass can be estimated using appropriate conversion factors. Such factors can be taken from the pertinent literature or experimentally determined for particular fungi of interest (*see* **Note** 8). The protocol described here was originally designed for the quantification of ergosterol during fungal growth on wheat straw [16] and may be adapted to also other fungi and substrates as appropriate.

1. Place the solid sample to be analysed (e.g. wheat straw colonised by fungi) onto preweighed filter papers and dry at 50°C for 48 h (*see* Subheading 2.4), until constant weight is achieved (*see* **Note** 9). Determine the dry weight of the sample as the difference between sample-containing and sample-free filter papers.

2. Homogenise the dry sample with the help of a ball mill at 40 oscillations for 5–10 min, and store homogenised samples in 15-mL Falcon® tubes (*see* Subheading 2.4) at ambient temperature and a dry and dark place.
3. Transfer 100 mg of the previously dried and homogenised sample into a 15-mL Falcon® tube, and add 1.0 mL distilled water, 0.5 g solid KOH, 5.0 mL methanol and 1.25 mL ethanol. After mixing, place the only loosely closed tubes in a water bath (70°C) and incubate for 30 min. During incubation, ensure thorough mixing by sufficiently shaking the tubes (*see* **Note** 10).
4. After cooling and settlement of the solids, transfer 4 mL of the supernatant to a 15-mL Falcon® tube, and add 2 mL of toluene containing 125 μM phenanthrene as an internal standard in addition (to correct for overestimations of ergosterol concentrations potentially resulting from evaporation losses of toluene). Extract ergosterol by horizontally shaking the tightly closed tubes for 1 h, using a horizontal shaker (*see* Subheading 2.4). After phase separation, transfer 0.5 mL of the toluene phase into a UPLC vial, and store at −20°C until ergosterol analysis by UPLC.
5. For UPLC analysis of ergosterol, samples (3.3 μL of the toluene phase after extraction; *see* above) can directly be subjected to an Acquity™ UPLC System (*see* Subheading 2.4) using a column temperature of 40°C. The following solvent system may be applied: (A) 10% methanol in deionised water (*see* Subheading 2.4), acidified to pH 3 with concentrated phosphoric acid, and (B) 100% methanol, acidified to pH 3 with concentrated phosphoric acid. Elution may be carried out using the following conditions: isocratic elution at 20% B for 0.14 min; linear increase to 100% B until 2.8 min; isocratic elution at 100% B until 3.2 min; linear decrease to 20% B until 3.25 min; and isocratic elution at 20% B until 3.5 min (0.5 mL/min flow rate). Recording a wavelength range from 220 to 400 nm is useful (detection wavelength: 278 nm). The method can be calibrated using external ergosterol (retention time about 2.88 min) and phenanthrene standards (retention time about 2.10 min).

4 Notes

1. *Cladosporium sphaerospermum* (syn. *Cladophialophora* sp. [17]) is not assigned to risk group 2 of microorganisms or higher according to German regulations (TRBA 460, assignment of fungi to risk groups) and is assigned to biosafety level 1

according to the CBS strains collection (http://www.cbs.knaw.nl/Collections) and ATCC (http://www.lgcstandards-atcc.org/). Nevertheless, C. *sphaerospermum* and related species have been reported to represent allergens being hazardous to patients with respiratory tract problems [17, 18] (https://microbewiki.kenyon.edu/index.php/). Therefore, readers interested in employing *C. sphaerospermum* or related fungi should acquaint themselves with the most recent information regarding the phylogenetic and taxonomic position of the fungus intended to be used and the applicable legislation concerning its risk group assignment, and follow the related safety measures to be applied.

2. Since (ball-)milled wheat straw or other suitable lignocellulosic (waste) materials may be obtained from various sources (e.g. agriculture, organic waste treatment facilities, certain research institutions), we recommend to search for a suitable supplier in your specific region. The straw obtained needs to be sterilised prior to use.
3. The toluene should be checked for evaporation losses from time to time and refilled if necessary.
4. Favourable media and further conditions such as incubation temperatures, times and agitation rates may be different for other fungi and hydrocarbons.
5. Due to different properties, other hydrocarbons may have to be applied at different concentrations and may require different co-solvents and/or detergents to ensure sufficient solubility. Also, other fungi may respond differently to pollutant hydrocarbons, co-solvents and detergents, which have to be considered for the successful design of an experiment.
6. Suitable UPLC and HPLC methods for the analysis of t-NP are described in Jahangiri et al. [19] and Junghanns et al. [4], respectively. Other pollutant hydrocarbons may require different analytical methods.
7. Further methods describing the establishment of mass balances from ^{14}C-labelled pollutant hydrocarbons are, e.g. described in Schlosser et al. ([20], Steffen et al. [21] and Steffen et al. [22]).
8. Factors enabling the conversion of fungal ergosterol contents into biomass and methods applied to determine such factors for fungi of special interest are, e.g. described in Pasanen et al. ([23], Montgomery et al. [24] and Klamer and Bååth [9]).
9. It is important to carry out drying until constant weight is achieved. Suitable drying temperatures and times may vary depending on individual conditions, which may be tested.

For instance, drying temperatures of 80 or 90°C and a drying time of 24 h are also frequently applied. However, a standardised protocol should be applied throughout once it has been fixed.

10. Mixing may be carried out, e.g. in 5-min intervals if executed by hand or continuously with the help of the shaking unit of a water bath if available.

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Oleaginous Microalgae Isolation and Screening for Lipid Productivity Using a Standard Protocol

Van Thang Duong, Boer Bao, and Peer M. Schenk

Abstract

Microalgae have great potential to serve as a feedstock for biodiesel, based on their high areal productivity and their ability to accumulate large amounts of triacylglycerols. In contrary to first-generation biofuel crops, microalgae do not need to compete with arable land or biodiverse landscapes and can be grown in virtually any type of water, including fresh, brackish, saline and wastewater. The use of local microalgal strains is desirable, as they are often dominant under the local geographical, climatic and ecological conditions. Here we describe a user-friendly method that enables the isolation of monoalgal strains, coupled to a standard protocol to evaluate their potential for lipid accumulation by directly comparing lipid productivities. Other important criteria to consider include the ability to harvest microalgae and extract their oil cost-effectively.

Keywords: Algae isolation, Biodiesel, Biofuel, Lipid productivity, Microalgae, TAG, Triacylglycerols

1 Introduction

Microalgae are widely distributed in varied environments. They can be found in all types of waters such as rivers, ponds, estuaries, ocean water and even sea ice. Microalgae are organisms that use sunlight and CO_2 for their photosynthesis to produce biomass. The growth and distribution of microalgae depend on the availability of sunlight and nutrient sources. Microalgae grow more abundantly in nutrient-rich environments where they can cause eutrophication. Eutrophication phenomena occur in nutrient-rich waters that may lead to negative impacts on living organisms in the ecosystems. The cell density of microalgae reaches a peak at optimum nutrient supply before others factors, such as light, become limiting. The following decay process by heterotrophic organisms (typically bacteria) uses large amounts of oxygen and causes shortage of oxygen content for other organisms, especially for aquatic animals. Nevertheless, microalgae that cause blooms are opportunistic and fast growing and therefore interesting to achieve high biomass

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productivities. Primary productivity is a parameter used for measuring the abundance of microalgae in the environment. Primary productivity is high in nutrient-rich environments, especially in tropical or subtropical waters and in abundance of solar irradiation. Environments such as coastal rock pools, estuaries and rivers that exhibit fluctuating conditions are more likely to harbour opportunistic and fast-growing microalgae [1, 2]. These robust microalgae are well adapted to extreme conditions and rapid changes. Their ability to survive is enhanced by being able to accumulate large amounts of lipids or starch. Thus, the chance of isolating promising fast-growing strains with the ability to accumulate large amounts of storage lipids from those environments is greater [3].

1.1 Criteria for Selecting Strains for Further Analysis

After obtaining pure microalgal isolates, all strains should go through prescreening and screening steps. Prescreening criteria cover essential features, such as the use of local strains that display rapid growth in the medium of choice, high salinity tolerance, the ability to accumulate large amounts of extractable lipids and to be cost-effectively harvested (e.g. by settling; Fig. 1). This is followed by a direct comparison of the lipid productivity of strains under standard and strain-optimized conditions. In addition, value-added products from the remaining biomass or the integration of other processes (e.g. wastewater nutrient removal) can be considered for economic reasons as part of a microalgae biorefinery concept [4].

One of the essential criteria for prescreening is whether the microalgal strains can grow in the desired medium, especially for large commercial cultivation. There is a great variety of media that can be used to culture microalgae such as F/2 medium [5] and Bold's basal medium [6]. However, the costs of these commonly used media are much higher than commercial fertilizer grade nutrients [7]. Thus, it is important to preselect species that can grow in the desired medium. This can already be done at the start of the procedure by adding fertilizers or desired nutrients into the collected water samples [8].

A further prescreening step is salinity tolerance. Saline water is the optimum sustainable water source for commercial microalgae cultivation as freshwater supply is limited when competing with land-based food crops agriculture [9]. In addition, increased salinity level can potentially induce lipid production and increase lipid content of some microalgal species [10]. Salinity can be greatly affected by the rate of evaporation and the quality of the available water source [11]. As water from cultivation continuously evaporates and is typically recycled, its salinity also increases over time. However, not all microalgae can tolerate high salt concentrations. *Dunaliella salina* is one of the extreme halophilic species that can even grow in a saturated salinity of 321 ppt [12, 13], whereas *Haematococcus pluvialis* cannot tolerate more than 10 ppt salinity [14]. Salinity level is just above 0 ppt (e.g. 4 ppt) for culturing freshwater species, 15 ppt for culturing brackish water species and 35 ppt for

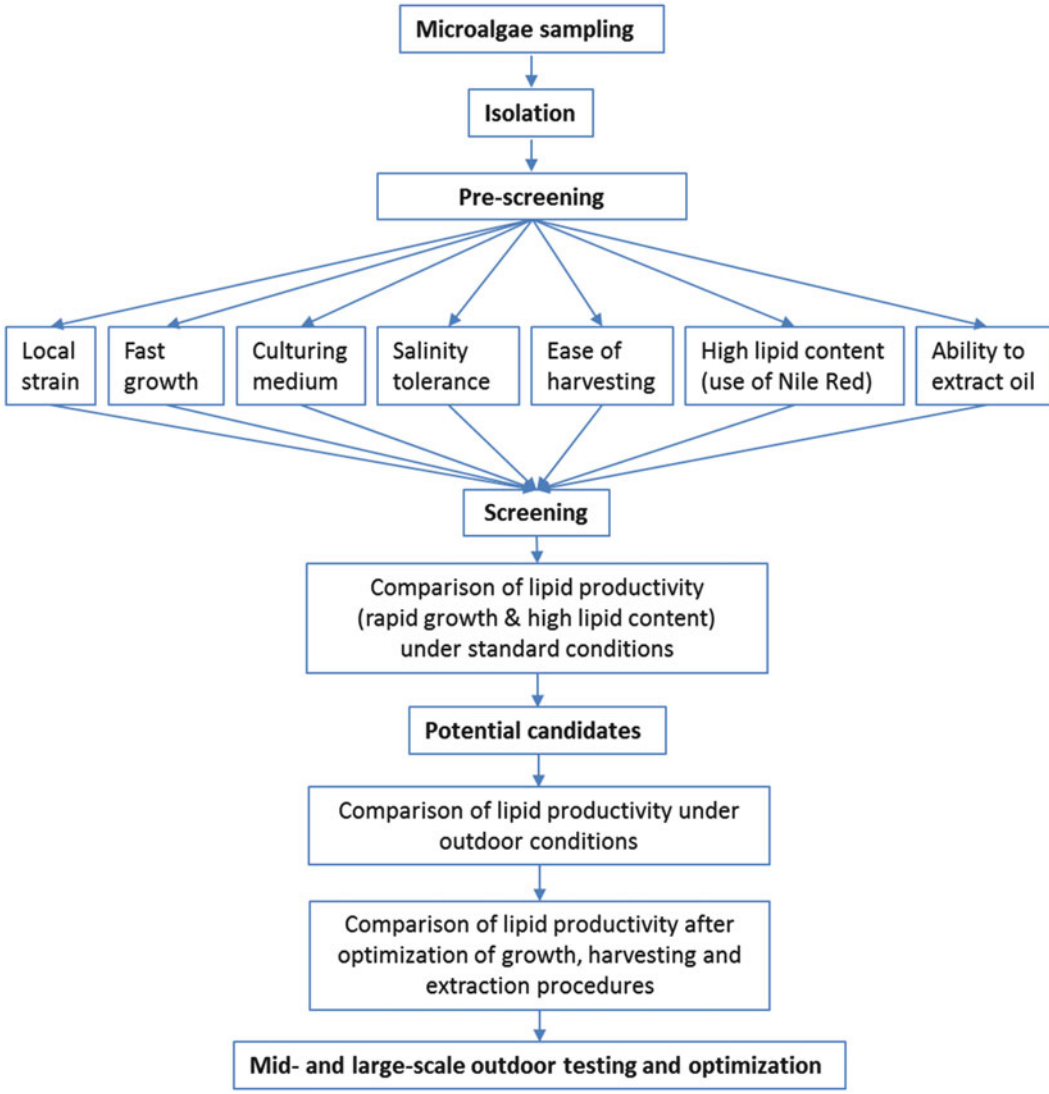

Fig. 1 Process for screening candidate microalgal strains for biodiesel production

culturing marine species. Salinity tolerance can be tested by culturing microalgae species at a range of salinities up to 35 ppt or higher.

Another important criterion when selecting strains is the harvesting method. There are several harvesting methods available such as centrifugation, filtration, flotation, flocculation and sedimentation. However, some of these techniques are energy consuming and attract high costs [15, 16]. Sedimentation is one of the most promising methods with low capital cost [16, 17]. It generally requires microalgae to have a relatively large cell size, colonial or filamentous morphology and high density in comparing to the medium [18]. The ability of settling can be tested by settling in a conical funnel for 24 h after cultivation and adjusting the

concentration to 1 g L^{-1}. The settling rate can be calculated by multiplying the volume of the cells with its concentration over time [19]. An alternative is to induce settling by inducing flocculation or by removing flagella from flagellate microalgae [20].

The ability to accumulate lipids can be tested by using the Nile red staining protocol shown below. After comparing lipid productivities using a standard protocol, suitable species and strains can be ranked and further optimized in a strain-specific manner before testing under outdoor conditions. Since outdoor environments and conditions are much more complex and difficult to control, it is important to test promising microalgal strains to see how they perform under conditions encountered in the field [8, 11].

2 Materials

2.1 Media for Isolation (Chemicals Provided by Merck)

2.1.1 F/2 Medium Developed by Guillard and Ryther [5]

1. $NaNO_3$ stock solution (1 L): $NaNO_3$ 75 g
2. NaH_2PO_4 stock solution (1 L): 5.0 g
3. Trace metals stock solution (1 L): Na_2EDTA 4.36 g, $FeCl_3 \cdot 6H_2O$ 3.15 g, 1 mL of each the five primary metals stocks below (make up five stocks separately):

   $CuSO_4 \cdot 5H_2O$ 1.0 g in 100 mL

   $ZnSO_4.7H_2O$ 2.2 g in 100 mL

   $CoCl_2 \cdot 6H_2O$ 1.0 g in 100 mL

   $MnCl_2 \cdot 4H_2O$ 1.8 g in 100 mL

   $NaMoO_4 \cdot 2H_2O$ 0.63 g in 100 mL
4. Vitamin stock solution (1 L) (keep covered in foil): biotin 10.0 mL of 0.1 mg mL^{-1} solution, vitamin B_{12} 1.0 mL of 1.0 mg mL^{-1} solution, thiamine HCl 0.2 g

2.1.2 Bold's Basal Medium (BBM) Developed by Bischoff and Bold [6]

1. $NaNO_3$ stock solution (1 L) 25 g
2. $CaCl_2 \cdot 2H_2O$ stock solution (1 L) 2.5 g
3. $MgSO_4 \cdot 7H_2O$ stock solution (1 L) 7.5 g
4. K_2HPO_4 stock solution (1 L) 7.5 g
5. KH_2PO_4 stock solution (1 L) 17.5 g
6. NaCl stock solution (1 L) 2.5 g
7. Alkaline EDTA stock solution (1 L): EDTA anhydrous 50 g, KOH 31 g
8. Acidified iron stock solution (1 L): $FeSO_4 \cdot 7H_2O$ 4.98 g, H_2SO_4 1.0 mL
9. Boron stock solution (1 L): H_3BO_3 11.42 g
10. Trace metal stock solution (1 L): $ZnSO_4 \cdot 7H_2O$ 8.82 g, $MnCl_2 \cdot 4H_2O$ 1.44 g, MoO_3 0.71 g, $CuSO_4 \cdot 5H_2O$ 1.57 g, $Co(NO_3)_2 \cdot 6H_2O$ 0.49 g

2.1.3 Agar Powder

2.1.4 Nile Red Powder: 9-Diethylamino-5-benzo [α]phenoxazinone ($C_{20}H_{18}N_2O_2$) Provided by Sigma-Aldrich. Concentration of Stock Solution in Ethanol: 250 μg mL^{-1} (Light Sensitive and Should Be Kept in Foil at 4° C).

2.1.5 Haemocytometer (e.g. Reichert Bright-Line Provided by Hausser Scientific)

3 Methods

Microalgae have diameters typically ranging from 2 to 50 μm and need to be initially identified morphologically under a microscope (*see* **Note 1**). Microalgae isolation methods include the use of microscopes for micromanipulation, single colony growth on agar plates, serial dilution and/or other supporting equipment or procedures such as flow cytometry.

3.1 Media Preparation

3.1.1 F/2 Medium

1. 950 mL of filtered seawater (using 0.2–0.3 μm filter paper).
2. Add 1.0 mL of $NaNO_3$ stock solution, 1.0 mL of NaH_2PO_4 stock solution, 1.0 mL of trace metals stock solution.
3. Top up to 1 L with filtered seawater.
4. Autoclave at 120°C for 20 min.
5. When the medium cools down to 40°C, add 0.5 mL of vitamin stock solution.
6. Store medium at 4°C.

3.1.2 BBM Medium

1. Prepare 936 mL of distilled water.
2. Add 10 mL of each six major stock solutions.
3. Add 1 mL of alkaline EDTA stock solution.
4. Add 1 mL of acidified iron stock solution.
5. Add 1 mL of boron stock solution.
6. Add 1 mL of trace metal stock solution.
7. Autoclave at 120°C for 20 min.
8. Store medium at 4°C.

3.1.3 Agar Medium

1. Before F/2 or BBM is autoclaved, add 15 g of agar (1.5% w/v). Shake lightly.
2. Autoclave at 120°C for 20 min.
3. Cool down the medium to 40°C (and add 0.5 mL of vitamin stock solution for F/2).
4. Pour about 20 mL of medium to sterilized Petri dishes in a laminar flow.
5. When the agar in dishes cools down, store at 4°C.

3.2 Micromanipulation Method

1. Place an inverted microscope and a micromanipulator in a laminar flow cabinet or a clean working environment. The micromanipulator can be a specialized instrument or can be simply prepared by pulling a micropipette on the flame of a Bunsen burner to a small diameter, approximately 3 to 5 times of the diameter of the microalgae to be isolated.
2. Prepare growth medium in 96-well tissue culture (microtiter) plates.
3. Place separated drops of medium on a slide that is placed on the inverted microscope.
4. Add a drop of sample containing microalgae to the first drop of medium on the slide (*see* **Note 2**).
5. Attach a micropipette with the micromanipulator or a silicone tube and suck a small amount of sterilized distilled water or medium in it.
6. Under the microscope or on the screen that is connected to the camera of the microscope, locate the single cell that needs to be isolated and suck up the single cell to the micropipette.
7. Transfer the single cell to a well of a 96-well tissue culture plate by blowing out.
8. If two or more cells are sucked up to the micropipette, transfer cells to the second drop of the slide and try to suck up a single cell and transfer it to a well. Repeat this step until you obtain a single cell.
9. When finished, wrap the plate with parafilm tape to prevent drying of the medium and place it in a culture room under light exposure from 120 to 200 μmol photons m^{-2} s^{-1}.
10. After 2 weeks, the culture can be observed under the microscope to check the growth and contamination.

3.3 Streaking on Agar Plates

1. Pour 1.5% agar medium to Petri dishes till half or two third full.
2. Lift the lid of the plate up slightly, just allowing enough space for putting a swab into the plate without risking contamination from the air.

3. Drop a small volume (e.g. approx. 100 μL) of the sample containing microalgae collected from the environment to one side of the plate. Use a sterile loop to streak the sample in a zigzag mode.
4. Sterilize the loop and hold it to cool down for a while. Streak another time in a different angle (*see* **Note 3**).
5. Repeat the above step three or four times.
6. Cover the lid, seal the plate, label the sample and incubate the plate in an inverted position at the appropriate temperature (e.g. room temperature) in the light from 120 to 200 μmol photons m^{-2} s^{-1}.
7. Check for the growth of colonies in two weeks under a microscope.

3.4 Serial Dilution

1. Dispense 9 mL of sterilized medium into each sterile tube.
2. Add 1 mL of the sample to the first tube. Mix well. Transfer 1 mL of the mix to the second tube. Repeat this step for the remaining tubes.
3. Incubate tubes at the appropriate temperature for two weeks at light exposure from 120 to 200 μmol photons m^{-2} s^{-1}. Check the monoalgal culture under a microscope. A monoalgal culture is usually obtained from dilution tubes 10^{-6} to tubes 10^{-10}. The method can also be carried out in microtiter plates with much smaller volumes.

3.5 Nile Red Staining

1. Add 3 μL of 250 μg mL^{-1} Nile red stock solution to 1 mL of samples and keep in foil.
2. The staining process takes around 20 to 30 min and can be applied to microalgal strains by using 96-well plates.
3. Measure fluorescent intensity on a fluorescence plate reader with an excitation wavelength of 530 nm and an emission wavelength of 575 nm [21, 22]. Measurement of fluorescence intensity for Nile red strained microalgae can be determined by flow cytometry [20], whereas the lipid droplets in microalgal cells can be observed under a fluorescence microscope as shown in Fig. 2 [3] (*see* **Note 4**).

3.6 Cell Sorting by Flow Cytometry

1. Prepare two separate samples: one Nile red stained tube and the other unstained tube. The stained sample is used to determine lipid content in microalgae and the unstained sample is used for sorting the high lipid content cells.
2. Turn on a flow cytometer. The analysis procedures depend on the particular instrument. However, the parameters should be controlled as follows: emission excitation intensity at 570 ± 25 nm [23, 24], pressure 31.4–31.5 psi, flow rate 15,000 to 20,000 cells s^{-1} [24].

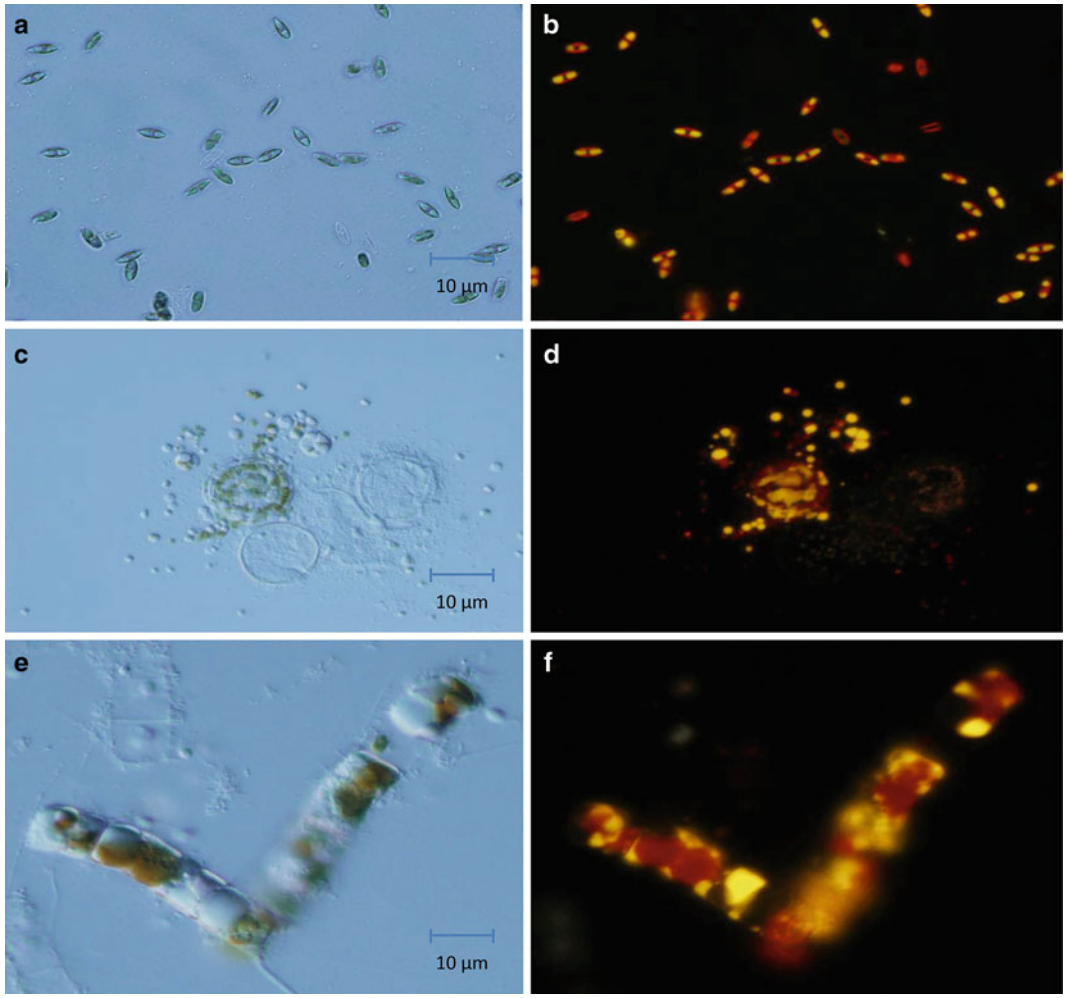

Fig. 2 *Navicula tripunctata*, *Cyclotella striata* and *Chaetoceros* sp. before (**a**, **c** and **e**, respectively) and after Nile red staining (**b**, **d** and **f**, respectively) under a fluorescence microscope. Yellow droplets show TAGs in lipid bodies and red shows autofluorescence from chlorophyll

3. Cells are sorted to 96-well plates containing 200 µL of culture medium per well. After sorting, the plate is labelled and cells are grown in a growth cabinet with light exposure of 120 to 200 µmol photons m^{-2} s^{-1} (*see* **Note 5**).

3.7 Growth Testing (Standard Protocol)

3.7.1 Inoculation

1. Add 1 mL of master culture to 150 mL medium in 250 mL flasks (*see* **Note 6**).
2. Connect flasks with a 1% CO_2 supply system (optional; *see* **Note 7**).
3. Grow microalgae in light intensity of 120–200 µmol photons m^{-2} s^{-1} over 4 to 5 days (*see* **Note 8**).

*3.7.2 Growth Experiment (See **Note 6**)*

1. Add 5 mL of inoculated culture to 150 mL medium in 250 mL flasks.
2. Connect flasks with a CO_2 supply system.
3. Grow in light intensity of 120–200 μmol photons m^{-2} s^{-1}.
4. Collect sample every 2 days to count cell density. Cell counting is conducted by using a haemocytometer.
5. Growth rate is calculated by the equation below [25] (*see* **Note 9**):

$$K' = \frac{\ln \frac{N2}{N1}}{t2 - t1}$$

where $N1$ and $N2$ = biomass at time 1 ($t1$) and time 2 ($t2$), respectively.

Doubling time can also be calculated once the specific growth rate is known:

$$\text{Doubling time} = \frac{\ln 2}{K'}$$

3.8 Starvation

1. After a fixed time of growth (early stationary phase), nutrients are depleted (removed by medium change with nitrogen- and phosphorus-free medium).
2. Incubate cultures for 3 more days in the light to induce lipid accumulation.
3. Collect samples for lipid analysis under GC/MS. The amount of biomass used for lipid analysis should be 10 mg dry weight (*see* **Notes 10** and **11**).

4 Notes

1. It is very difficult to make axenic algal culture and most cultures are non-axenic with the presence of some bacteria. However, contamination from other eukaryotic microorganisms should be avoided during the isolation process, and the final culture should be monoalgal.
2. Micromanipulation is the conventional method of choice that is still very popular for microalgae isolation. The method was initially used by Bakoss [26] for cloning single cells of leptospires. Then, Sherman [27] used a mechanical micromanipulator to separate spires from a yeast ascus cluster. The use of micromanipulation for microorganism isolation is reliable and has been greatly improved. Microalgae can be examined under the microscope at 400× to 1,000× magnifications. An inverted microscope that is connected to a camera can greatly support visualization of the cell to be handled. Moreover, commercial

micromanipulators are equipped with a pressure device to support picking up single cells more precisely. Normally, microalgae samples collected from natural environments contain a wide range of organisms including zooplankton, phytoplankton, bacteria and other microorganisms. Samples of seawater may need to be concentrated by filtration before isolation because of the low density of microalgae cells in the water. Thus, based on the nature of samples, concentration and filtration can be applied to enable the efficient use of a micromanipulator.

3. Streaking on agar plates is a popular method used in microorganism research to obtain single-cell-derived colonies. This method can potentially obtain individual cells from the sample. Single cells from the sample can be separated on agar surface and grow up to colonies. Streaking patterns can be as simple as zigzag for low density samples or more complex as the 3 streak quadrant for high density samples. The plate method requires a sterile or sanitized environment near the flame of a Bunsen burner. Streaking requires delicate skills to make sure that cells are not damaged. A good streaking plate is where single cells are separated and grow up to a separated colony. However, it should be noted that the majority of microalgal strains do not grow on standard agar plates or grow very slowly.
4. Microalgae produce triacylglycerides (TAGs) as storage lipid in cells. Lipid extraction requires separation of the remaining biomass from oil for quantification [28]. Conventional microalgae lipid extraction methods are widely based on solvent extraction and gravimetric determination [29]. From the current study of microalgae lipid extraction, different methods of lipid extraction have been developed by using different catalysts to enhance extraction efficiency such as using different solvents, sonication, direct saponification and supercritical CO_2 extraction [30]. Fatty acids are determined by gas chromatography/mass spectroscopy (GC/MS) to profile suitable fatty acids components for biodiesel production. Lipid extraction and profiling are time-consuming and cost-ineffective. Preliminary screening of lipid-producing microalgae is the first step to determine lipid quantification of microalgae. Nile red is a lipid soluble fluorescence dye and has been used to stain triacylglycerols in animal and microbial cells [21].The efficiency of Nile red straining depends on the varied structure and thickness of the cell wall and the cytoplasmic membrane of microalgae. Chen et al. [21] further developed the Nile red staining method by optimizing the binding ability of the dye to intracellular neutral and polar lipids using different staining conditions such as Nile red concentration, temperature, staining

time, the solvent dimethyl sulfoxide (DMSO) and cell concentration. The results showed that the best staining efficiency was obtained at a staining condition of 0.5 μg mL^{-1} of Nile red dye at 40°C for 10 min, a DMSO concentration of 25% (v/v) and cell concentrations ranging from 5×10^4 to 4×10^5 cells mL^{-1} [21].

5. Along with conventional isolation methods described above, an automated single-cell isolation method is developed and nowadays widely used for cell sorting known as flow cytometry. The procedure may vary depending on particular flow cytometry producer. However, the principle of this method is based on different channels of light scatter angles and fluorescence intensities that allow the distinction of different clusters of cells and other heterogeneous particles [31]. Flow cytometry has been successfully used for single-cell isolation of microalgae from waters with many different microalgal strains [3]. The method cannot easily be used for fragile microalgae, such as dinoflagellates, as damage to single cells can easily occur [32]. However, pressure adjustment can minimize the impact of cell damage on microalgae during cell sorting.
6. Growth testing of microalgae is important for screening potential strains for biofuel production. Growth rates of each microalgal strain are different and change based on changes of environmental conditions, especially the availability of nutrients in the culture. Nitrogen and phosphorus are the main nutrients for microalgae cultivation. The concentrations of those compounds in the medium vary from 1,500 to 3,000 μM and 100 to 200 μM for nitrogen and phosphorus, respectively. The ideal final ratio of N:P for the best growth of microalgae is considered 30:5 [33, 34]. Mineral compounds are also necessary to add to the medium for the growth of microalgae. Apart from trace elements, potassium is required in relatively large amounts, and diatom cultivation requires silica as the main chemical source for cell wall construction. The growth of microalgae can be visualized by a growth curve that goes through different phases, starting with the lag phase, followed by an exponential growth phase, a declining or stationary growth phase and a death phase. For the production of biodiesel from microalgae, the exponential and stationary phases are important because they are related to biomass and lipid productivity. The duration of the exponential phase depends on the health of the microalgae, their growth rate, the size of the inoculum, culturing conditions and the capacity of the medium and growth vessel.
7. CO_2 is needed for microalgae growth and supplying of CO_2 will promote growth. However, depending on specific circumstances, CO_2 supply (e.g. from a gas bottle or flue gas) is not

necessary because microalgae can assimilate CO_2 from the environment.

8. It is important that the microalgae culture used for inoculation before performing the growth test is healthy and recently grown. Microalgae used for the inoculation culture are collected from master cultures. The inoculation culture also requires nutrient-rich medium that promotes growth to reach the exponential phase. This is an important step to ensure that microalgae transit from inactive stage from the master culture to the active stage in growth experiments.
9. Growth rate is a parameter that is used to assess the growth capacity of individual microalgae strains. Cell counting or cell concentration measurement between the first and last day of the steep exponential phase is necessary for identifying maximal growth rate. Table 1 shows some examples of typical microalgal growth rates and lipid contents reported in the literature.
10. Starvation of microalgae in stationary phase is known as a period of lipid accumulation in microalgal cells [35, 36]. Nutrient and light availability plays a crucial role for the growth of microalgae. Light or nutrient starvation starts at the early stationary phase of the microalgal growth life cycle. Under nutrient shortage conditions but in the presence of light, the biochemical compounds produced by photosynthesis are stored as starch or fatty acid in the form of TAGs as

Table 1
Growth rate and total lipid content of some microalgal strains

Species	Growth rate (day^{-1})	Lipid content (% dry weight biomass)	References
Achnanthes sp.	0.99	44.5	[37]
Botryococcus braunii	–	25.0–75.0	[16]
Chaetoceros muelleri	–	33.6	[16]
Chaetoceros sp.	0.87	16.3	[37]
Chaetoceros sp.	0.74	13.1	[38]
Cryptomonas sp.	0.33	21.4	[38]
Isochrysis sp.	0.81	20.7	[38]
Nannochloris sp.	–	20.0–35.0	[39]
Nannochloropsis sp.	0.62	42.4	[37]
Rhodomonas sp.	0.35	12	[38]
Skeletonema costatum	0.95	9.5	[37]

Table 2
Increase of microalgal lipid contents under conditions of nutrient shortage [10]

Microalgal species	Nutrient stress	Changes in lipid profile after induction
Chlamydomonas reinhardtii	Nitrogen limitation	Increase in total lipids
Scenedesmus subspicatus	Nitrogen limitation	Increase in total lipids
Nannochloropsis oculata	Nitrogen limitation	Total lipid increased by 15.31%
Chlorella vulgaris	Nitrogen limitation	Total lipid increased by 16.41%
Phaeodactylum tricornutum	Nitrogen limitation	TAG levels increased from 69 to 75%
Dunaliella tertiolecta	Nitrogen limitation	Five times increase in lipid fluorescence
Scenedesmus sp.	Nitrogen and phosphorus starvation	Lipids increased 30% and 53%, respectively
Chlorella kessleri	Phosphorus limitation	Increase in unsaturated FAs
Chlamydomonas reinhardtii	Sulphur limitation	TAG was increased by twofold
Cyclotella cryptica	Silicon starvation	Increased in total lipids from 27.6% to 54.1%

energy reserves under these adverse conditions [10]. Nutrient starvation for lipid induction in microalgae is widely used for biofuel production in many microalgal strains but other stresses may also lead to cellular TAG accumulation [10]. Table 2 shows examples of the increase of lipid contents under nutrient starvation stress.

11. While biomass growth and lipid accumulation are both important, it is the lipid productivity (amount of lipid per day and cultivation volume or area) that matters the most. A standard protocol has therefore been developed that first inoculates a pre-culture from the master cultures before this saturated culture is used to start the standard growth experiment. The experiment is carried out for several microalgal strains in parallel using a minimum of three biological replicates (separately inoculated cultures). The method is described in detail by Lim et al. [36] and Fig. 3 shows a schematic overview. After selecting potential candidate microalgal strains, these then need to undergo further evaluation under outdoor conditions and after careful individual optimization for each strain (Fig. 1). Finally, larger-scale production needs to be tested where cultivation, harvesting and oil extraction are taken into consideration.

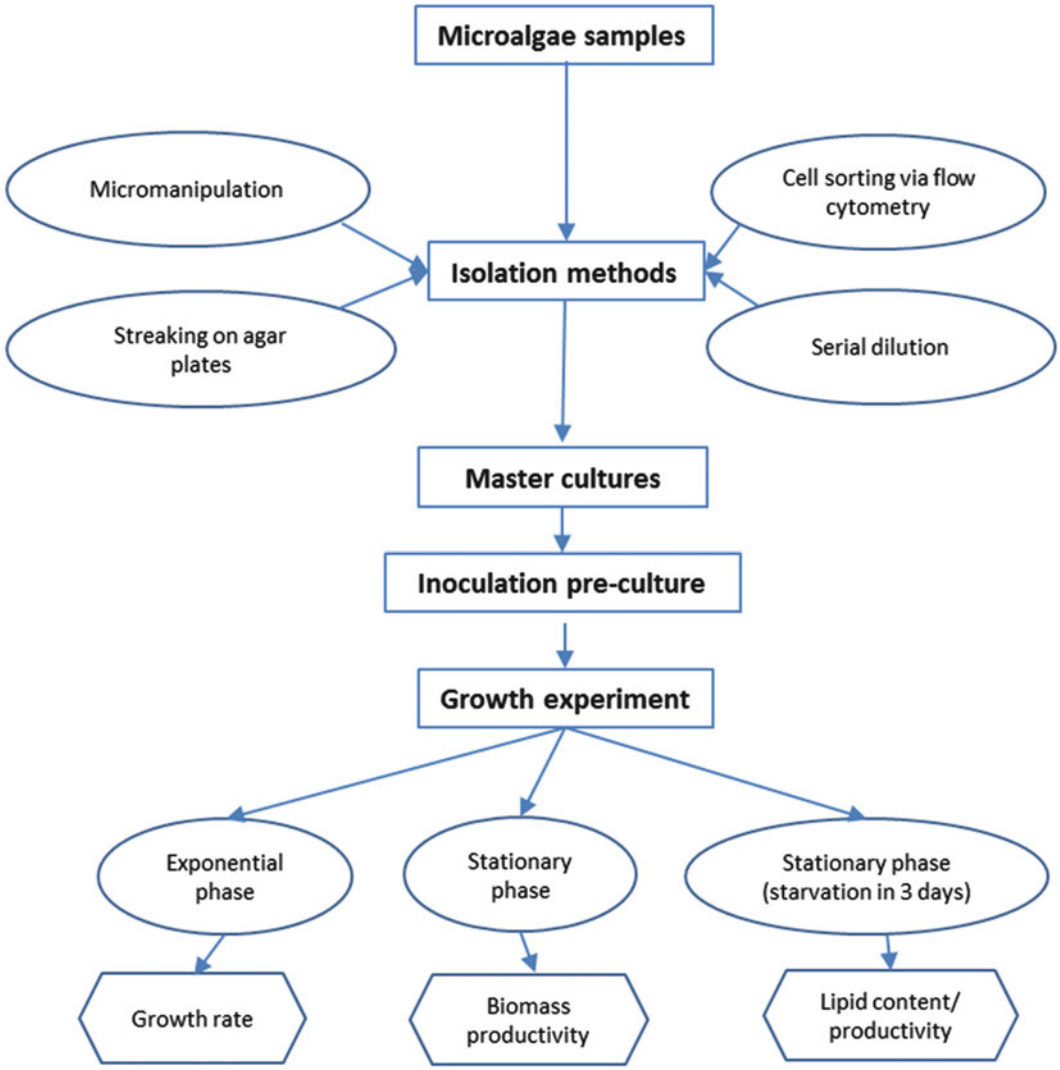

Fig. 3 Standard protocol for growth testing of microalgae for biofuel production (for details *see* [36])

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Preservation of Microbial Pure Cultures and Mixed Communities

Bram Vekeman and Kim Heylen

Abstract

Microorganisms are valuable and irreplaceable resources for scientific research and biotechnological innovation and should be safeguarded. Therefore, systematic preservation of isolated pure cultures, enriched mixed cultures, or environmental samples should become an integral part of good research practice. Cryopreservation of biological material is a low-tech, widely applicable way of long-term and stable storage. Its success is mostly dependent on the cryoprotective agent, used to protect cells from mechanical injuries due to ice formation, the stability of the freezing temperature, and the correct manipulations before and after storage. Although cryopreservation success can be organism dependent, the protocol described here proved successful for various fastidious pure and mixed cultures when frozen at −80°C using 5% (v/v) dimethyl sulfoxide as cryoprotective agent. Numerous parameters of the protocol can be changed or optimized, and guidelines are given to develop a custom-made cryopreservation protocol.

Keywords: Complex carbon, Cryostorage, Freezing, Resuscitation

1 Introduction

Archiving of biological material is a very important aspect of microbiology and microbial ecology as microbial pure cultures, communities, or original environmental samples are often irreplaceable (1–4). Stable and long-term storage of microbial resources (a) allows validation of previously obtained results, (b) avoids accidental contamination or losses during research, (c) catalogs genetically identical biodiversity for future research, and (d) ensures long-term accessibility of well-documented cultures well after their discovery for biotechnological or commercial use. Despite the general consensus on its importance and in contrast to data archiving, robust storage of biological material is often neglected and still not widely incorporated in good research practices (4).

Preservation of microbial pure cultures is most common. Long-term storage is most often achieved by lyophilization or

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cryopreservation in liquid nitrogen or in freezers at −80°C (5). Lyophilization is mainly limited to specialized labs or culture collections because of specialized equipment but offers advantages over cryopreservation such as ease of storage and transport (6). Many parameters may influence the success of preservation (6); therefore, long-term preservation is considered an empirical research field, where it is very difficult to compare or apply data from literature. Unfortunately, when standard freezing of a glycerol stock (commonly at 10–15% (v/v)) at −20°C or −80°C is unsuccessful, one generally resorts to storage at 4–15°C in appropriate conditions (i.e., medium and headspace) and periodical subcultivation. Storage in a cold room is not a "stable" long-term preservation method and does not guarantee identical biological material after several years. Subcultivation is a time-consuming process and can easily result in contamination or genetic drift. In addition to the abovementioned advantages to systematically preserve biological material, another major incentive is the increasingly strict rules of journal and funding agencies to deposit described pure cultures in a public culture collection, available for the scientific community upon request (7, 8). This type of open source might become mandatory with science performed by public funds. However, researchers are often unaware that they are obliged to provide the suitable preservation conditions of their pure cultures upon deposit in public culture collections (8), and deposits might be refused when this information is not available.

Environmental or applied research on bacterial cocultures, enrichment cultures, consortia, or even environmental samples approximates the real biological in situ conditions more than pure culture work and thus is generally deemed more relevant. However, experimental work cannot be reproduced or verified because cultures or samples are not stored in a stable manner and batch or continuous cultivation in any kind of reactor will result in a different community composition. In addition, the risk of losing biological material due to calamities such as power failures or contaminations is higher than with pure cultures, with no backup material available in private or public repositories. Research on storage of these kinds of samples is in its infancy. Still, the limited reports available demonstrate that their stable cryopreservation is possible using similar simple protocols as for pure cultures (9–13). Embedding of cells in a matrix such as exopolysaccharides in aggregates or biofilm-covered particles in soils or sediments provides extra protection to the cells during preservation and is a major advantage compared to planktonic pure cultures. However, for mixed communities or samples, not only specific activities need to recover after preservation, but also the composition of the revived community, both species richness and evenness, should be identical to the original. Still, maintained functionality and community structure after cryopreservation have already been

successfully demonstrated for denitrifying biomass of a fluidized, methanol-fed denitrification reactor (10), a methanotrophic coculture, an oxygen-limited autotrophic nitrification/denitrification biofilm, and fecal samples from a human donor (13). These examples should encourage further preservation attempts of mixed communities and environmental samples to provide backup biological material for future research. Furthermore, although public culture collections currently do not have appropriate quality control tools at their disposal to handle these types of samples, public deposits of non-pure cultures will become increasingly relevant in the near future.

It is widely recognized that different bacterial species and even strains within one species can exhibit great variability in the success rate of different preservation conditions (14). Thus, it is almost impossible to give an overview of recommendations for different groups of bacteria. But general trends are obvious, for example, the more challenging, albeit successful, preservation of marine strains, probably due to salt stress during freezing (12, 15). We refer to reference works such as *The Prokaryotes* and *Bergey's Manual of Systematic Bacteriology*, which, next to detailed information on isolation and growth, also include sections on maintenance and preservation for functionally or phylogenetically related groups. Despite the well-known organism-dependent nature of preservation, the wide applicability of a very simple cryopreservation protocol was recently demonstrated for a broad range of fastidious microorganisms and mixed communities (12, 13, 15–17). Nevertheless, a preservation procedure should always be verified before long-term storage. Therefore, this chapter presents a simple generic cryopreservation protocol that can be carried out at any research facility when revival of biological material after freezing in a glycerol stock fails. It also includes tips and tricks for the development of a custom-made cryopreservation protocol. Instead of a comprehensive overview, parameters presented here should be regarded as a starting point for a nonspecialist trying to safeguard its bacterial "work horses."

2 Materials

2.1 Pre-preservation

1. Suitable cultivation media for culturing the organism of interest (specific media information is available from bacteria culture collections or in scientific literature). Media are always best used when freshly prepared, but many can be stored at 4°C for up to 3 months.
2. Serum vials, serum flasks, Erlenmeyer flasks, or Schott bottles for cultivation in liquid media (Duran, www.duran-group.com or VWR, www.vwr.com).

3. Gray butyl rubber stoppers (aerobic incubation) (VWR, www.vwr.com), red bromobutyl rubber stoppers (aerobic and anaerobic incubation), or black butyl rubber stoppers (anaerobic incubation) (Rubber bv, www.rubberbv.nl), aluminum crimp seals, and a bottle crimper (VWR, www.vwr.com) (black butyl stoppers excrete toxic compounds in the media that are inhibitory for most cultures. Make sure to boil the black butyl rubber stoppers up to 2 times for 30 min to remove/reduce the toxic compounds/effect or use the red variant if possible).
4. Trehalose (Sigma, www.sigmaaldrich.com).
5. Bacto™ Tryptic Soy Broth (TSB), Dehydrated (BD diagnostics, www.bd.com).

2.2 Cryopreservation

1. Cells from late log or early stationary growth phase.
2. Dimethyl sulfoxide (DMSO) (Sigma, www.sigmaaldrich.com). Use gloves when handling DMSO as it is quickly absorbed into the body through the skin and may transport harmful substances into the body with it.
3. 0.2-μm nylon filters (Sartorius, www.sartorius.com) or Teflon® polytetrafluoroethylene (PTFE) syringe filter (Sigma, www.sigmaaldrich.com). Make sure to prewash the PTFE filter with alcohol and rinse with DMSO.
4. For concentrating of sensitive cells: polycarbonate filters 0.2 μm (Millipore, www.millipore.com) and polycarbonate syringe filter holder (Sartorius, www.sartorius.com) (*see* **Note 3**).
5. Preservation containers: Nunc Cryotube™ cryogenic vials (Thermo Scientific, www.thermoscientific.com), Eppendorf tubes (Eppendorf AG, www.eppendorf.com), polypropylene screw-top Falcon tubes (TPP, www.tpp.ch), Microbank™ tubes (Pro-Lab diagnostics, www.pro-lab.com), glass serum vials (VWR, www.vwr.com), and Schott bottles (Duran, www.duran-group.com) (*see* **Note 10**).
6. −80° freezer or liquid nitrogen tank.
7. Nunc CryoFlex™ tubing, (Thermo Scientific, www.thermoscientific.com) (*see* **Note 12**).

2.3 Resuscitation

1. Warm water bath at 37°C
2. Eppendorf tubes (Eppendorf AG, www.eppendorf.com) or polypropylene screw-top Falcon tubes (TPP, www.tpp.ch)
3. Precooled centrifuge at 4°C
4. Fresh liquid or solid culture medium

2.4 Evaluation of Cryopreservation Success and Quality Control

2.4.1 Quantitative Viability Measurements

Adenosine Triphosphate (ATP) Determination

1. BacTiter-Glo™ Microbial Cell viability assay (Promega, www.promega.com).
2. rATP stock (10 mM) (Promega, www.promega.com).
3. White Nunc C8 LockWell LumiNunc MaxiSorp (Thermo Scientific, www.thermoscientific.com).
4. GloMax®96 microplate Luminometer (Promega) with built-in orbital plate shaker for complete reagent mixing in 96-well plate.
5. Always wear gloves to avoid contamination from human skin. Skin contains ATP!

Live/Dead Flow Cytometry Counting

1. 5 mM Na_2EDTA solution (Sigma, www.sigmaaldrich.com).
2. CytoCount™ (Dako, www.dako.com).
3. Live/dead stain: 30 mM propidium iodide (Invitrogen, www.invitrogen.com) mixed with SYBR®Green (10^{-2} dilution in DMSO) at a 1:50 ratio. The staining solution should be stored in the freezer and be protected from the light.

2.4.2 Plate Counts and Purity Tests

1. Polystyrene or glass petri dishes (Novolab, www.novolab.be)
2. Solidifying agents: agar, agarose (BD diagnostics, www.bd.com), or Gelzan™ (Sigma-Aldrich) gellan gum (Sigma, www.sigmaaldrich.com)
3. Complex organic media used for detecting heterotrophic contaminants in oligotrophic cultures (e.g., 1/10 trypticase soy agar, nutrient agar R2A, etc.) (Oxoid™, www.thermoscientific.com or BD diagnostics, www.bd.com)

2.4.3 Extinction Culturing

1. *See* Sect. 2.1 pre-preservation in materials.
2. Miniaturized extinction culturing in 96-well microtiter plates (Novolab, www.novolab.be).

3 Methods

Cryopreservation is the process of stabilizing biological material at very low temperatures. Many parameters influence the success rate of a specific cryopreservation method. The two most important are cryoprotective agents (CPAs) and storage temperature. Freezing of biological material is normally lethal, and CPAs are added to cell suspensions before freezing to ensure a higher cell survival rate than in its absence. CPAs reduce the solution effects that play during freezing by inducing osmosis (i.e., movement of water) and diffusion (i.e., movement of solutes). Although glycerol is the best known and most widely used CPA, mainly because of its low toxicity and ease of handling (exposure time and washing are less

critical, see further), a pairwise comparison of its cryoprotective effectiveness in cryomicrobiology showed it to be less effective than DMSO (18). To allow long-term stable cryopreservation, samples are ideally stored at temperatures below the glass transition state of water (−137°C) to prevent the presence of liquid water. When stored at higher freezing temperatures, small temperature changes in the surrounding, even briefly, can result in dramatic temperature shifts in the preserved material with recrystallization of water to ice and potential destruction of cells due to cryoinjuries (19).

Besides choice of CPA and stable freezing temperatures, our experience with fastidious microorganisms and mixed communities suggests that suboptimal manipulation of cultures before and after freezing is the major reason of decreased viability after storage. Cryopreservation should be successful for most organisms if these simple guidelines are followed, as also thoroughly discussed by Tindall (20): (a) use correct equilibration time and temperature according to CPA used, (b) store biomass at the lowest possible stable temperature, and (c) thaw preserved biomass quickly and immediately remove CPA upon thawing. For non-fastidious bacteria, viability loss of a large fraction of preserved cells as a result of not following these guidelines is compensated by the high cell numbers that were initially stored. But these specific guidelines could be vital for preservation of limited biomass of fastidious cultures.

The following protocol is a generic cryopreservation protocol applicable for pure or mixed cultures and recommended when relevant information on preservation of your organism(s) of interest is lacking. When this standard protocol fails, we suggest varying different preservation parameters using the elaborate test protocol described in Sect. 3.4. However, it speaks for itself that specific requirements for manipulation and growth – each microbiologist is most familiar with his own pet organism – should also be taken into account for preservation.

3.1 Pre-preservation Growth

Early stationary (or late exponential) phase is the ideal growth phase for preservation of a pure culture, because the cells encounter depletion of necessary nutrients and high cell density, switch to a starvation strategy, and express new proteins increasing resistance to changing conditions. Performing simple growth curves of pure cultures and cell harvest in this growth phase prior to preservation increases the success rate of preservation (*see* **Note 1**). When possible, high cell concentrations ($>10^8$ cell/ml) are preserved on the premise that the majority of cells die during long-term storage, but a sufficient number will survive to enable the continuation of the strain:

1. Choose a suitable liquid medium for your organism(s) of interest.

2. Fill serum vials, serum flasks, or Schott bottle to 20% capacity with liquid medium, sterilize, and inoculate with a small amount of sample.
3. Seal the serum vials with appropriate stoppers and adapt headspace if necessary.
4. Incubate cultivation flasks at an optimal growth medium, either on a shaker or in static conditions depending on the preference of your organism(s) of interest.
5. Follow growth over time with optical density measurements at 600 nm using a spectrophotometer.
6. Harvest cells at late exponential/early stationary phase by taking 1 to 100 ml of culture (*see* **Note 2**) and concentrate biomass if necessary (*see* **Notes 3–5**).
7. Put at 4°C for max 30 min (*see* **Note 6**). Continue with the cryopreservation ASAP.

3.2 Cryopreservation

1. Prepare a sterile DMSO stock. DMSO must be sterilized by filtration using a 0.2-μm nylon syringe filter or a Teflon® PTFE syringe filter which has been prewashed with alcohol and rinsed with DMSO (*see* **Note 7**).
2. Prepare fresh liquid growth medium.
3. Prepare the preservation medium by adding 10% (v/v) DMSO to fresh liquid growth medium mix thoroughly and keep at 4°C (*see* **Note 8**) until use.
4. Add the chilled harvested cells to an equal volume of cooled preservation medium containing 10% (v/v) DMSO (to obtain a final concentration of 5% DMSO (v/v)) (*see* **Notes 9–11**).
5. Transfer the cell suspension to cryogenic vials (*see* **Note 12**) suitable for the total preservation volume. Nunc Cryotube™ cryogenic vials are most convenient. Generally 0.5–1.0 ml is added to each container (for the standard total volume of 1 ml, this is 500 μl culture and 500 μl liquid medium with 10% DMSO (v/v)).
6. Equilibrate for 15 to 30 min (*see* **Note 13**) at 4°C, if possible while shaking or stirring.
7. Freeze the cryovials at temperatures equal or lower than −80°C (*see* **Notes 14** and **15**).

3.3 Resuscitation

The final step in the preservation protocol is thawing the preserved biomass in order for cells to reconstitute, become active, and reproduce:

1. Thaw cryovials in a warm water bath at 37°C (*see* **Note 16**). Do not let vials warm up to 37°C, but process the vials immediately upon thawing.

2. Transfer the complete culture to sterile Eppendorf tubes for centrifugation (15 min, 6,000 g) at 4°C (*see* **Note 17**).
3. Remove supernatant and wash pellet twice with fresh growth medium to remove all DMSO (*see* **Note 18**).
4. Transfer the culture to serum vials with fresh growth media and incubate in the appropriate conditions.
5. Foresee extended incubation time. The cells will demonstrate a prolonged lag phase for recovery of growth and activity (*see* **Note 19**).
6. Confirm the authenticity of the culture before using it in other experiments. Any suitable, in-house technique can be used (*see* **Note 20**).
7. All preserved cells from one vial are used for resuscitation. So, if growth and/or activity is successful, make sure to again preserve a subculture. Depending on the volumes and purpose of preservation, at that stage, several aliquots can be made and preserved (*see* **Note 21**).

3.4 Development of Custom-Made Cryopreservation Protocol

If the generic protocol fails for your organism(s) of interest, many parameters can be adapted or altered in the development of a custom-made cryopreservation protocol. The difficulty is the choice of parameter(s) to adapt, especially as the solution might be a combination of adjusted parameters. Unfortunately, prediction of actual success of a specific CPA and its optimal concentration for the cryopreservation of a specific microorganism is very difficult because the exact nature of cryoinjury and its prevention is often unknown and can vary across organisms. Novel protocols always need to be evaluated experimentally. Here, we describe some tips and tricks to get started:

1. Stick to DMSO as CPA. Generally, it is the best performing CPA (*see* **Note 22**).
2. Test different concentrations of DMSO (*see* **Note 23**).
3. If the growth medium of your organism of interest is a defined medium, add 1% trehalose (w/v) and 0.3% trypticase soy broth (w/v) to the preservation medium (*see* **Notes 24** and **25**), in addition to DMSO (*see* **Note 26**).
4. If the growth medium is a defined medium and your organism of interest does not consume complex carbon substrates, add 1% trehalose (w/v) alone or in combination with 0.3% trypticase soy broth (w/v) to the pre-preservation growth medium (*see* **Note 27**). However, first do growth-inhibition tests and growth curves in this amended growth medium. Addition of complex substrates to the preservation medium and the pre-preservation growth medium can be done in combination or separate (*see* **Notes 28** and **29**).

5. Define quality control tools for the comparison of preservation success rate of different protocols. For this, take extra cell culture at time of cell harvest and after resuscitation and apply appropriate techniques. Depending on your interest, qualitative evaluation of growth, quantitative evaluation of culturability via cell enumeration (plating or (miniaturized) extinction culturing), quantitative evaluation of viability with flow cytometric live/dead counts, or ATP monitoring can be used. For some microorganisms, measurement of specific activity rate is a more logical proxy for growth, and recovery of specific activity rate after preservation can then be directly correlated to the success rate of the applied preservation protocol (*see* **Note 30**):
   (a) For miniaturized extinction culturing:
      – Prepare dilution series from 10^{-2} up to 10^{-10} (or higher depending on the initial density of the culture used) in a sterile 96-well plate (min 100 and max 200 µl/well).
      – Incubate the plate at the optimal growth temperature and adjust headspace if necessary.
      – Monitor growth at different time points by measuring the optical density at 600 nm with a spectrophotometer.
      – Calculate most probable numbers by using most probable number tables (21).
   (b) For live/dead flow cytometry counts:
      – Dilute the culture to an optimal cell density (10^4–10^6 cells/ml).
      – Add 10 µl ml^{-1} live/dead staining solution, 5 mM Na_2EDTA, and 10 µl ml^{-1} CytoCount™.
      – Mix the samples thoroughly and incubate in the dark for 15 min at room temperature.
      – Samples can be analyzed using a flow cytometer, e.g., a CyAn™ High Performance Flow Cytometry (DakoCytomation, Belgium) (*see* **Note 31**).
      – Analyze the data using appropriate software (e.g., Summit™ 4.3 software).
   (c) For ATP determination (*See* **Note 32**):
      – Prepare 1 µM ATP in culture medium (100 µl of 1 µM ATP solution contains 10^{-10} moles ATP).
      – Prepare 10-fold serial dilutions of ATP in culture medium (1 µM to 10 pM; 100 µl volumes would contain 10^{-10} to 10^{-15} moles of ATP). This serves as standard curve.

- Add 100 µl of the different serial dilutions of ATP or microbial cell culture to each well of a White Nunc C8 LockWell LumiNunc MaxiSorp 96-well plate.
- Prepare control wells with cell-free medium to obtain a value for background luminescence.
- Equilibrate the plate and its contents to room temperature.
- Add 100 µl of BacTiter-Glo™ reagent (*see* **Note 33**) to each well.
- Mix briefly on an orbital shaker and incubate for five minutes (*see* **Note 34**).
- Record luminescence with a luminometer (*see* **Note 35**).

6. Even if the generic protocol was successful, adaptations to the resuscitation can increase cultivability of preserved cells and shorten the lag phase (*see* **Note 36**).
7. Once a good working cryopreservation protocol is obtained, standardization is essential. Because of the complexity of preservation, small variations can lead to changes in the preservation success. Standardization of the protocol will assure a higher consistency and comparability between preservation results.

4 Notes

1. Mixed communities and enriched and natural environmental samples have already been successfully preserved without taking into account the growth phase of its microbial members (9, 10, 12, 13).
2. For dense cultures ($OD_{600nm} > 0.75$) 500 µl to 1 ml will suffice. Volume size can be dependent on the reasons for storage, chosen recipients, organization of freezer, or liquid nitrogen tank. From an enrichment of *Candidatus* Kuenenia stuttgartiensis, a 2-l volume was harvested from an active continuous reactor, concentrated to 500 ml, cryopreserved, and resuscitated to successfully seed a 2-l continuous reactor, resulting in immediate growth. This proof of principle suggests that any kind of volume can be cryopreserved if the presented protocol is followed.
3. Cell numbers from non-dense cell cultures can be increased through settling of biomass, centrifugation, or filtration. Appropriate methods should be chosen based on the sensitivities of the organism(s) of interest, but often centrifugation will be the most straightforward. Centrifugation of cells is generally

performed at 6,000 g for 15 min to avoid damaging of the cells prior to preservation. However, lower centrifugation rates can be applied for longer time to concentrate the cells. Sensitive strains can be concentrated onto a polycarbonate filter using a polycarbonate syringe filter holder.

4. Alternatively, cells can also be harvested from solid plate media and suspended in fresh growth medium prior to preservation. For most bacteria, including aerobic methane oxidizers, this has already proven successful.
5. For strict anaerobes, make sure to do these manipulations in an anaerobic chamber. For enrichments of anaerobes *Candidatus* Kuenenia stuttgartiensis, *Candidatus* Scalindua sp. (12), and *Candidatus* Methylomirabilis oxyfera (Heylen, Ettwig, Jetten, unpublished), manipulations were successfully performed under atmospheric conditions.
6. Growing cells at lower temperatures can improve the preservation success through the expression of cold-shock proteins (22).
7. Cryoprotective agents should be prepared in aliquots for single use to minimize the risk of contamination and moisture introduction with repeated use from one container.
8. The temperature at which equilibration is performed depends mostly on the level of toxicity of the applied CPA. For DMSO, toxicity is lower at 0–5°C (18), and equilibration for this CPA is therefore standardly performed at 4°C.
9. Always add equal volumes of cell culture and medium with double concentration of CPA together. This avoids cell death prior to preservation due to the toxic shock after addition of a small volume (e.g., 100% DMSO to a cell culture).
10. A concentration of 5% (v/v) DMSO as cryoprotectant generally allows successful preservation, as was confirmed by work with aerobic and anaerobic ammonium oxidizers, aerobic and anaerobic nitrite-dependent methane oxidizers, aerobic nitrite oxidizers, enrichment cultures, and environmental samples. However, for some organisms the optimal DMSO concentration might be higher or lower.
11. Some CPAs, for example, DMSO, are toxic, and their addition to cell cultures can lower the preservation success of sensitive cultures. For most planktonic cells addition of a CPA is required to cryopreserve viable cultures. However, for mixed communities that grow in flocs or biofilms, the matrix in which cells are embedded might provide sufficient protection against freezing injuries of the cells (9). For these cultures, it should be tested if CPA addition results in better, equal, or worse recovery rate after preservation. For enrichments of

Candidatus Kuenenia stuttgartiensis (12) and *Candidatus* Methylomirabilis oxyfera (Heylen, Ettwig, Jetten, unpublished), a methanotrophic coculture and an OLAND biofilm (13), the use of DMSO improved the specific activity recovery rate. The activity recovery of cryopreserved fecal material from human donor was not influenced by DMSO addition (13). Nevertheless, the initial community structure was better preserved after freezing with DMSO, as determined by 16S rRNA gene sequencing.

12. Any kind of recipient suitable for the applied freezing temperatures can be used. We have already successfully used Nunc Cryotube™ cryogenic vials, Microbank™, glass serum vials, Eppendorf Tubes®, polypropylene screw-top Falcon tubes, and Schott bottles. Please note that glass has a higher thermal conductivity than polypropylene, which is most often used for the production of plastic cryovials. This might result in higher cooling and warming rates within the vial, decreasing the chance of cryoinjury, and is especially advised when preserving bigger volumes. Microbank™ is a convenient, ready-to-use system designed to greatly simplify the storage and retrieval of bacterial cultures. It comprises of a unique cryovial system incorporating treated beads and a special cryopreservative solution with unknown composition. It can have a broad applicability, but this remains to be demonstrated for fastidious cultures.

13. Good use of CPAs requires knowledge of their rate of penetration. This means that permeable CPAs such as DMSO require an equilibration time between 15 and 30 min to penetrate the cell wall and cell membrane and equilibrate intracellular solutes before the cell suspension can be frozen. Slow permeable CPAs like glycerol need between 30 and 60 min. Semipermeable CPAs only penetrate the cell wall but not the cell membrane (e.g., mono- and disaccharides, amino acids, polymers with low molecular weight). Other CPAs, i.e., polymers with a high molecular weight (such as proteins, polysaccharides, and others; see (18)), are non-permeable and only protect the cells from external ice formation and thus do not require equilibration time.

14. In general, cryoinjury is the result of traversing a lethal temperature zone at approximately −15 to −60°C (at least twice when freezing and thawing). For long-term storage, the use of temperatures below −137°C (i.e., glass transition temperature of pure water), at which all water is solidified, is recommended (19). For storage in freezers, it is extremely important to limit temperature variations. The relative position of the vial in the freezer and subjection to heat transmission through door or walls can have an effect on the freezing pattern. Therefore, it

is advised to have a dedicated freezer for long-term storage (>6 months) of biological material, but note that ultralow temperatures in liquid nitrogen containers are most suitable (23), with reports of successful storage up to 35 years (which is the longest that tests have run). In liquid nitrogen containers, cryovials are best stored in the gas phase (± −180°C) just above the liquid nitrogen (−196°C). Storage in the liquid phase can result in the entrapment of liquid nitrogen inside the vial leading to a possible explosion or biohazard release. Furthermore, liquid nitrogen penetration inside vials might also be a source of contamination of other submerged samples. If storage in the liquid nitrogen phase is required, make sure to properly protect the vials using Nunc CryoFlex™ tubing.

15. The duration of cryopreservation with successful growth and activity recovery depends on the storage temperature and, most importantly, the stability of the storage temperature. We found that evaluation of cryopreservation success after one to six weeks of storage compared to one year was identical (15, 16). Therefore, a short cryopreservation test can quite accurately predict the success of long-term cryostorage.
16. Very small amounts of intracellular ice are compatible with recovery. Nevertheless, slow warming allows crystals to recrystallize and to grow (24). Therefore, it is essential to rapidly warm cryopreserved material after storage, so there is insufficient time for recrystallization, and the ice simply melts. Make sure to use a warm water bath with an active circulation of the water to avoid deviation of the temperature in the immediate vicinity of the cryovial. Never use incubators at 37°C as this will significantly prolong thaw time.
17. To minimize the risk of contamination during resuscitation, disinfect the external surface of the vial by wiping with ethanol prior to opening.
18. Some cultures might be tolerant to high concentrations of DMSO (up to 100 nM). These cultures, upon thawing, might be immediately transferred to fresh liquid medium or plated on fresh solid medium, without removal of DMSO.
19. The prolonged lag phase results from chemical and temperature stress to which cells were subjected during cryostorage.
20. For pure cultures, first check purity via microscopic investigation of the resuscitated cell suspension or, if possible, a simple streak on the appropriate solid growth medium to confirm the presence of a single cell and colony type, respectively. Subsequent sequence analysis of the 16S rRNA gene or other housekeeping genes (25, 26) from a single colony is the most straightforward way to demonstrate the correct identity of the

preserved bacteria, taken into account that contamination with highly related strains is very unlikely. For authentication of mixed communities, denaturing gradient gel electrophoresis (DGGE) (27), fluorescent in situ hybridization (FISH) (www.arb-silva.de/fish-probes/fish-protocols/), or 16S rRNA amplicon sequencing (13) is appropriate.

21. A seed lot system might be preferred to enhance the continuity and longevity of the biological material over time (28). The principle is that a working stock and a seed stock are prepared. Ideally the seed stock is preserved separately from the working stock. When the working stock is depleted, a vial is retrieved from the seed stock to prepare a new working stock. The last seed stock vial is then used to prepare a new seed stock. The second seed stock remains only one or two passages from the original material, but may be separated by many years.
22. Many other CPAs exist that might be more suitable for specific organisms. We refer you to two very comprehensive reviews by (18, 29). Furthermore, the combination of different CPAs, with different chemical structure, permeability rate, and corresponding mode of action, can also have a synergistic effect. Also note that planktonic cells of various nitrite oxidizers could be successfully preserved without CPA (15).
23. Cryopreservation of various nitrite oxidizers showed little difference in activity recovery after cryopreservation with 1%, 5%, or 10% DMSO (v/v), except for *Nitrospina gracilis* 3/211 for which activity only recovered after preservation with 10% DMSO (v/v) (15).
24. Complex substrates (e.g., yeast extract, blood serum, and peptones), sugars, or alcohols (e.g., sucrose and mannitol, respectively) can also function as CPA (18, 30). Because most cryopreservation research has been performed on heterotrophs, systematically grown on complex media, the cryoprotective role of complex substrates was an often neglected feature in cryopreservation of oligotrophic microorganisms. These carbon compounds are either taken up by the cell to avoid intracellular ice formation, only penetrating the periplasmic space where they stabilize the cell membrane during ice formation by interaction with the polar head groups of the phospholipids (i.e., disaccharides such as trehalose and sucrose) or remain extracellular and only protect the cells from external ice formation.
25. Growth and/or activity recovery after cryopreservation was repeatedly shown to be significantly improved when using the combination of 5% (v/v) DMSO, 1% trehalose (w/v), and 0.3% trypticase soy broth (w/v) (combination of the latter two was termed TT) compared to sole use of 5% (v/v) DMSO

for planktonic cells of oligotrophic cultures (12, 15–17), aggregates of enriched and mixed communities (12, 13), and environmental samples (13). The combination of trehalose, which is known to protect cells against desiccation effects, and TSB as well as their respective concentrations was originally arbitrarily chosen (16). Other (combinations of) complex carbon compounds thus might give better results. For nitrite oxidizers, the addition of 1% sucrose showed no improvement (15), except for *Nitrospina gracilis* 3/211 for which it was essential when using DMSO concentrations other than 10% (v/v).

26. DMSO can also be left out. Complex carbon sources in the preservation medium can also be used as sole CPA (15).
27. For nitrite oxidizers, pre-preservation growth in 0.3% TSB (w/v) increased the activity recovery after cryopreservation by more than 60% (15).
28. Besides the necessity of these extra tests, another big drawback of the addition of complex substrates to pre-preservation growth medium is the possible overgrowth of background flora in mixed communities. To that end, only 1% trehalose (w/v) was added to pre-preservation growth medium for *Candidatus* Kuenenia stuttgartiensis and *Candidatus* Scalindua (12) and *Candidatus* Methylomirabilis oxyfera (Heylen, Ettwig, Jetten, unpublished). However, with subsequent sole use of 5% DMSO (v/v) as CPA, activity recovery was comparable to the use of 5% DMSO (v/v) + TT as CPA without additions to the pre-preservation growth medium.
29. Preservation induces a viable but non-culturable state (VBNC) in a significant amount of preserved cells (16), making the recovery of culturability and not viability most problematic after cryopreservation. The addition of TT to the preservation medium significantly reduced the fraction of VBNC cells of various methane oxidizers.
30. Work on nitrite-oxidizing bacteria has demonstrated that monitoring of optical density, ATP, or activity rate produced interchangeable data on viability recovery (15).
31. Optimal settings for each stain should be determined before measuring (31).
32. All steps are performed at room temperature (22–25°C). Equilibrate the lyophilized BacTiter-Glo™ substrate and BacTiter-Glo™ buffer to room temperature before use. Mix both reagents together by gently vortexing, swirling, or inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo™ substrate should go into solution easily, in less than one minute. Finally equilibrate reagent at room temperature for at least 15 minutes before use.

33. Store the lyophilized BacTiter-Glo™ substrate and BacTiter-Glo™ buffer at −20°C for long-term storage. During frequent use the buffer can also be stored at 4°C or at room temperature for 48 h without loss of activity. Reconstituted BacTiter-Glo™ reagent (buffer + substrate) should be used within eight hours when the reagent is stored at room temperature, four days at 4°C, one week at −20°C, and one month at −80°C with less than 20% loss of activity.
34. The incubation time is required for the lysis of the cells and the release of ATP.
35. Instrument settings depend on the manufacturer. An integration time of 0.25 to 1 second per well should serve as a guideline.
36. Supplementing the standard growth medium with filter-sterilized spent medium (i.e., supernatant of actively growing culture) or cell-free extracts (32), precursors of important biochemical pathways, alternative energy and carbon sources or more generally cyclic AMP, homolactones (*N*-acyl homoserine lactones) (33, 34), or short peptides (34) have already been shown to efficiently increase cultivability of "unculturable" bacteria. Extended incubation after resuscitation (five weeks instead of two) increased the cultivability of various methane oxidizers by 50% (16).

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Erratum to: Protocols for High-Throughput Isolation and Cultivation

Karsten Zengler

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The original version of this chapter was inadvertently published with an incorrect author group. The correct sole author is Karsten Zengler.

Further, the original chapter was published without an Acknowledgement. The correct information is given below:

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The original article was corrected.

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Maria Genovese, Renata Denaro, Daniela Russo, Francesca Crisafi, Santina Santisi, Simone Cappello, Laura Giuliano, and Michail M. Yakimov

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The original version of this chapter was inadvertently published with incorrect spelling of surname of the last author. The name should read Michail M. Yakimov, and not M. Yakimov Michail.

The original article was corrected.

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