In the following chapter, we discuss the role of two-phase substrate transfer and partitioning for its bioavailability. As an example, 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).
Two-Phase Cultivation Techniques for Hydrocarbon-Degrading Microorganisms

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Abstract

In the following chapter, we discuss the role of two-phase substrate transfer and partitioning for its bioavailability. As an example, 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).

1 Introduction

Actively growing microbes are typically surrounded by water, and 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 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 adjust) 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 bacteria 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 (Zengler et al. 2002).

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 [7] within which hydrocarbon transport...
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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

\[
\frac{Q}{t} = A \cdot v_{tr} \cdot \text{Driving force}
\]

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 interphase rather than an interface 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 \([8]\). 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 interphase layer, attachment of cells to second-phase hydrocarbons and subsequent biofilm formation is an efficient microbial adaptation to promote substrate bioavailability, i.e. to increase the aqueous concentration-gradient and subsequent microbial growth rates \([9, 10]\) on liquid, solid, and nonaqueous phase liquid (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.

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 (≤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 procedures aims at controlling the substrate fluxes to metabolically active cells of poorly water-soluble substrates such as the polycyclic aromatic hydrocarbon, anthracene (cw,sat ≈ 40 μg 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.
167. 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).

168. Addition of the inoculum to the cultivation bottle and incubation at the desired temperature on a rotary shaker at 100–150 rpm.

169. 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 [11, 12] 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 [13]:

(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 (Holliger et al. 1993). Adaptations of the protocol described below have also been used for the enrichment of bacteria dechlorinating trichlorobenzene and other chlorinated compounds (Holliger et al. 1992).

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
257. 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 (Holliger et al. 1993)) and gas phase (N₂/CO₂ or H₂/CO₂). 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 (N₂).
   (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 (Reineke and Knackmuss 1984; Spain and Nishino 1987; [14]).

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. (1974). See Note XII.
   (b) Install an aeration system allowing aeration at rates between 3 and 15 l_air l⁻¹ medium 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. 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.

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 (van Udden 1967). 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 [15].

VI. Aqueous steady-state hydrocarbon concentrations are normally established within a few hours [16].

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 [13, 17].

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 [14].

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.

References

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