

A systematic study of the influence on microbial kinetics of the presence of an organic phase during microbial isolation

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The present study was conducted to isolate hexane degrading strains with low affinity constant (k_s) values in order to solve limitations by microbial activity during odorous volatile organic contaminants (oVOCs) treatment in two-phase partitioning bioreactors (TPPBs). For this purpose, microbial isolation was carried out using an activated sludge inoculum in presence of a non-aqueous phase (2,2,4,4,6,8,8, heptamethylnonane, HMN) with a high affinity for hexane in order to maintain low hexane aqueous concentrations. The kinetic parameters (μ_{max} and k_s) of 3 strains isolated (2 in absence and 1 in presence of HMN) and of a hexane degrading *Pseudomonas aeruginosa* BM-B-450 strain (to serve as biotic control) were determined. The bacterial strains isolated in absence of HMN exhibited similar values to those obtained for the bacterium isolated in the presence of HMN (μ_{max} ranging from 0.11 to 0.20 h⁻¹ and k_s from 0.26 to 0.42 mg l⁻¹). In addition, these values were comparable with the values recorded for *P. aeruginosa* BM-B-450. Therefore, the results herein obtained suggest that the addition of an organic phase during the isolation process does not result in the isolation of k_s strategic bacteria (bacteria with a high affinity for the substrate).

Keywords: hydrophobic VOCs, isolation, k_s , microbial kinetics, TPPBs.

Introduction

Biological methods are considered the most cost-efficient and environmentally friendly option for the treatment of large flow rates of air contaminated with low concentrations of odorous volatile organic compounds (oVOCs) (Van Groenestijn and Hesselink, 1993; Hodge and Devinny, 1995). Unfortunately, the performance of these processes is mainly restricted by the high hydrophobicity of some specific oVOCs (such as alkanes, terpenes), which limits pollutant transfer from the gas to the aqueous phase and therefore oVOC biodegradation (Devinny et al. 1999). The addition to a biological process of a second non-aqueous phase (NAP) with a high affinity for the target hydrophobic oVOCs can enhance their mass transfer to the aqueous phase. These systems, called two-phase partitioning bioreactors (TPPBs), provide a higher driving

force for mass transfer and induce an increase in the gas interfacial area, which ultimately improve the transfer of hydrophobic oVOCs to the aqueous phase (Quijano et al. 2009). Therefore, higher elimination capacities (ECs) can be obtained with this technology. For instance, α -pinene ECs 10 times higher than those recorded in a similar system without a NAP were observed by Muñoz et al. (2008) in a stirred tank bioreactor supplied with 10% of 2,2,4,4,6,8,8, heptamethylnonane (HMN). However, the potential of this technology can be affected by limitations in microbial activity. In this sense, Hernández et al. (2010) observed that the enhancement in the transfer of hexane in a stirred tank bioreactor supported by the presence of 20 % of silicon oil 200 cSt was attenuated by limitations in microbial activity, as shown by the fact that the ECs recorded during continuous hexane biodegradation were far lower than the maximum hexane transfer capacity recorded under abiotic conditions. In addition, Fazaelipoor (2007) developed a mechanistic model that showed that the presence of silicone oil did not increase toluene biodegradation performance in the case of microorganisms with large affinity constants (k_s) (low affinity of the microorganism for the oVOCs). Thus, bacteria with high k_s values will grow (and therefore degrade the oVOCs) at very low growth rate in TPPBs since the high ECs achieved in these systems induce very low oVOCs aqueous phase concentrations. For this reason, TPPBs should be operated with low constant affinity strains. The present study was therefore conducted to obtain hexane degrading strains with low k_s values. For this purpose, the isolation was conducted using an activated sludge inoculum in presence of HMN in order to maintain low hexane aqueous concentrations during isolation.

Materials and Methods

Isolation of hexane-degrading bacteria

Glass flasks of 120 ml were filled with 18 ml of a minimum mineral salt medium (MSM) according to Díaz et al. (2008) and inoculated with 2 ml of activated sludge from Valladolid sewage work in the presence and absence of 10 % of HMN. The flasks were closed with butyl septa and sealed with aluminium caps. The systems were supplied with increasing amounts of hexane (5 μ l during the first 10 days, 10 μ l from day 10 to 25 and 15 μ l during the last 11 days, resulting in initial hexane headspace concentrations of 34, 67 and 101 g m⁻³, respectively) and periodically re-inoculated with the 2 ml of the previous culture. The isolation procedure was carried out in duplicate (namely systems A and B) for approx 36 days under orbital agitation (250 rpm) at 30 °C. The determination of hexane degrading bacteria was performed by spreading serial dilutions of liquid samples from each flask (under sterile conditions) on agar plates (20 g l⁻¹) under selective conditions (hexane atmosphere) at 30 °C. After 2 days of growth, colonies with different shapes and sizes were picked and transferred into new agar plates under similar cultivation conditions in order to obtain well isolated strains.

Determination of microbial kinetic parameters

The k_s and the maximum specific growth rate (μ_{max}) of the bacteria above isolated were determined in a series of experiments carried out in gas-tight 1250 ml bottles supplied with 250 ml MSM and varying headspace hexane concentrations in duplicate (25, 50, 100 and 150 μ l of hexane). The systems were inoculated with the tested bacteria at an

initial OD₆₅₀ of 0.01 and the microbial kinetics parameters were determined by periodically monitoring the CO₂ and hexane headspace concentration for approx 24-36 h. A series of tests carried out with a hexane degrading *Pseudomonas aeruginosa* BM-B-450 strain was also performed to serve as biotic control.

Analytical procedures

Hexane quantification was performed by gas chromatography (Hewlett-Packard 6890, Palo Alto, USA) coupled with a Mass Spectrometer Detector (Hewlett-Packard 5973 MSD, Palo Alto, USA) according to Hernández et al. (2010). CO₂ and O₂ concentrations were determined in a gas chromatograph (Varian CP-3800, Palo Alto, CA, USA) coupled with a thermal conductivity detector according to Hernández et al. (2010). Biomass concentration was determined via optical density measurements at 650 nm using a HITACHI U200 UV/visible spectrophotometer (Hitachi, Tokyo, Japan).

Results and Discussion

Isolation of hexane-degrading bacteria

As a result of the isolation process, several colonies were obtained in presence and absence of HMN. In total, 7 different colonies were obtained, however, only the large morphologies were able to grow in liquid medium: system A without HMN (HMN_A), system B without HMN (HMN_B), and system A with HMN (HMN_A).

Determination of microbial kinetic parameters

A simple Monod kinetic was chosen to determine the microbial kinetic parameters of these 3 isolated bacteria and *P. aeruginosa* BM-B-450 in order to compare them. The use of a Monod model was justified by the low concentration range used in this work. Bacterial growth in the exponential phase can be defined by the next expression:

$$\frac{dX}{dt} = \mu X \quad (1)$$

where X and μ represent the biomass concentration and the specific growth rate, respectively. The values of μ can be obtained by integrating equation (1). Due to the fact that microbial kinetics were determined during the initial stages of the biodegradation process, where hexane concentration was not significantly degraded, X could not be measured accurately. However, microbial growth was recorded by monitoring CO₂ concentration [CO₂] since X and [CO₂] are directly related in the exponential phase and the values of [CO₂] obtained from GC-TCD measurements are very sensitive and reliable. Therefore X can be calculated by the next expression:

$$X = \frac{[CO_2]}{Y_{CO_2/X}} \quad (2)$$

Where Y_{CO₂/X} represents the CO₂/biomass yield coefficient.

From equations (1) and (2) the following expression for the determination of μ can be obtained:

$$\frac{d[CO_2]}{dt} = \mu[CO_2] \quad (3)$$

Thus, by representing the logarithm of the ratio of outlet and inlet $[CO_2]$ ($[CO_2]_f$ and $[CO_2]_i$, respectively) versus the elapsed time, the specific growth rate can be estimated from equation (4):

$$\ln \left[\frac{(CO_2)_f}{(CO_2)_i} \right] = \mu t \quad (4)$$

The values of μ at different hexane aqueous concentrations for the 3 strains isolated and *P. aeruginosa* BM-B-450 are depicted in figure 2. It must be noted that the $[CO_2]$ data used for the determination of μ corresponded to an interval in which the hexane concentrations remained similar to the initial values.

The Monod equation derived for each bacterium was capable of describing the experimental μ for the range of hexane aqueous concentrations here studied, which support the confidence in the values of μ_{max} and k_s estimated (Figure 2).

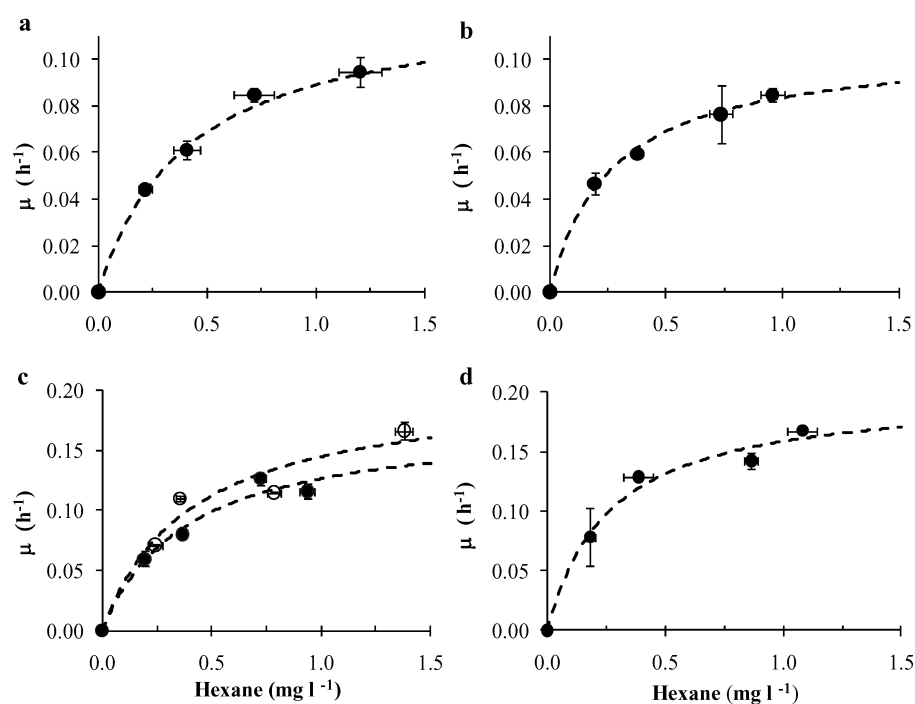


Figure 2. Experimental (circles) and theoretical (dashed line) influence of hexane concentration on the specific growth rate of HMN_A (a), HMN_B (b), HMN_A' and HMN_A'' (c) and *P. aeruginosa* BM-B-450 (d). “” represents a replicate done to assess the reproducibility of the methodology used.

According with a Monod kinetic, specific growth rate is defined as:

$$\mu = \frac{\mu_{\max} \cdot C_{aq}}{k_s + C_{aq}} \quad (5)$$

where C_{aq} represents the hexane aqueous concentration in equilibrium with the hexane headspace concentration. The kinetic parameters μ_{\max} and k_s from equation (5) were determined using the Solver tool of Excel (Microsoft Corp., USA). The kinetics parameters obtained for the 4 tested strains are showed in table 1.

Table 1. Kinetic parameters

Bacteria	μ_{\max}	k_s
With HMN		
$^{+}\text{HMN}_A$	0.20 ± 0.02	0.40 ± 0.07
$^{+}\text{HMN}_A'$	0.18 ± 0.02	0.39 ± 0.12
Without HMN		
$^{-}\text{HMN}_A$	0.13 ± 0.01	0.42 ± 0.01
$^{-}\text{HMN}_B$	0.11 ± 0.01	0.27 ± 0.01
<i>P. aeruginosa</i> BM-B-450	0.20 ± 0.03	0.26 ± 0.14

The bacterial strains isolated in absence of HMN exhibited similar μ_{\max} and k_s values to those obtained for the bacterium isolated in the presence of HMN (μ_{\max} ranging from 0.11 to 0.20 h⁻¹ and k_s from 0.26 to 0.42 mg l⁻¹) (Table 1). In addition, these values were comparable with the experimental values obtained for the biotic control conducted with *P. aeruginosa* BM-B-450. The mode of substrate uptake of bacterial strains isolated in presence of HMN could explain the results of this study. Hence, the presence of NAP droplets could have supported the direct contact between the hydrophobic cells and NAP droplets, which would have favored the exposure of bacteria to higher hexane concentrations, contrary to what would be desired (Bouchez-Naïtali et al. 1999).

Conclusions

Therefore, the results herein obtained suggest that the addition of an organic phase during the isolation process does not result in the isolation of k_s strategic bacteria (bacteria with a high affinity for the substrate). In this context, future research must be devoted to isolate bacteria in presence of NAPs but without direct contact between the cells and the NAP droplets.

Acknowledgements

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