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Characterization of the biological conversion of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in direct micellar systems

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Abstract

The whole cell biological conversion of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene by the *E. coli* JM109(pPS1778) recombinant strain carrying the naphthalene dioxygenase and regulatory genes cloned from *Pseudomonas fluorescens* N3 in micellar systems has been investigated using biochemical and chemico-physical techniques. Reverse and direct micellar systems have been tested. Non-ionic surfactants (Tween and Triton X series) were found not to inhibit either the growth of the bacteria and the expression of the hydroxylating dioxygenase enzyme in such systems and were utilized in order to speed up the naphthalene conversion by increasing its solubility and also its bioavailability. The phase behavior of the direct micellar system was characterized through light scattering and other chemico-physical techniques. Further addition of isopropyl-palmitate 1–2% v/v to the micellar systems resulted in an increase of the apparent substrate concentration in solution and particularly its bioavailability thus allowing faster catalytic conversions resulting in an increase in productivity for the process. Since the *cis*-dihydrodiols are acquiring considerable potential as chiral pool synthons in asymmetric synthesis for a variety of industrial processes, possible applications for efficient small and large-scale production of such compounds are discussed.

Keywords: Naphthalene; (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene; Naphthalene dioxygenase; Compartmentalized biosystems; Direct micellar systems; *E. coli* JM109(pPS1778) recombinant strain; *Pseudomonas fluorescens* N3

1. Introduction

Surfactant aggregation in water and organic media is a well established research area. The physico-chemical properties of colloidal phases L_2 (reverse micelles, water in oil microemulsions) and L_1 (direct micelles, oil in water microemulsions) have been thoroughly elucidated during the last few decades by means of scattering techniques, such as photon correlation spectroscopy, small angle X-ray and neutron

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scattering [1], or introducing photochemical or paramagnetic probes [2]. Such studies have allowed characterization of the phase behavior over a wide range of compositions and temperatures for ionic and non-ionic surfactant systems. In the last two decades, in addition to this basic physico-chemical characterization of self-assembled surfactant aggregates, the potential applications of micellar solutions have been exploited [3]. In fact, micellar solutions are microheterogenous media in which solvophobic compartments are present. Such pockets represent a microenvironment with different solubilization characteristics from the bulk medium. These compartments are separated from the continuous medium by a surfactant monolayer film, whose spontaneous curvature, H_0, determines the phase behavior.

The potentialities of solubilizing proteins in reverse micelles has been investigated in some detail [4]. Various studies have been focused on the build-up and optimization of chemical 'microreactors' where the water-insoluble substrate is dissolved in the continuous phase of 'water in oil' microemulsions and subsequently converted by enzymes stabilized in the aqueous 'droplet' of the microemulsions [5].

Conversely, the utilization of micellar solutions for biodegradation processes as a tool to increase the solubility and therefore the bioavailability of insoluble pollutants is still very limited compared with the reverse micelle enzymology area [6–9]. It has been shown that microorganisms such as *Escherichia coli*, *Candida pseudotropicalis* and *Acinetobacter calcoaceticus* can survive in some water in oil microemulsions; the water contained in the microemulsions being sufficient to maintain the cells viable for short periods of time [10–17].

The enzymes involved in the biodegradation processes of hydrocarbons, and particularly aromatics, present in natural or engineered microorganisms, could have large biotechnological developments both in terms of environmental detoxification and production of fine chemicals [18,19,40–43]. In the case of aerobic biodegradations, oxygenases catalyze the regio- and stereo-selective hydroxylation of a large variety of hydrocarbons, often giving products with an enantiomeric purity difficult or impossible to obtain with traditional synthetic methods [18,40–43]. The substrates of such enzymes have frequently very low solubilities in water systems, therefore, in order to increase the reaction rates and optimize the product yields it is necessary to devise systems with enhanced solubilities of hydrophobic compounds where the catalyzed reaction can still occur under optimal conditions [20].

The aim of this work is the study of whole-cell bioconversion in microstructured systems such as direct or inverse micellar solutions and the understanding of the different factors which control the bioavailability of water-insoluble substrates in the micellar phases. Such objective is indispensable for the design of an efficient chemical bioreactor for the production of *cis*-dihydrodiols starting from insoluble aromatic hydrocarbons like naphthalene.

2. Experimental

2.1. Materials

Tween 20, Tween 60, Tween 85 were kindly supplied by Bregaglia (Italy). Triton X100 and Triton X114, isopropyl-palmitate were purchased from Fluka (Switzerland). Water was purified in a reverse osmosis Milli-Ro system and subsequently treated with a Milli-Q system. All the other chemicals were of the best purity available.

2.2. Bacteria

The *E. coli* JM109(pPS1778) recombinant strain carrying naphthalene dioxygenase and regulatory genes cloned from *Pseudomonas fluorescens* N3 was used for the bioconversion studies [21]. This system is able to catalyze the conversion of naphthalene and other aromatic

compounds to the corresponding *cis*-dihydrodiols derivatives as previously reported [22–25]. In the case of naphtalene, the absolute configuration and the enantiomeric purity of the product (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene were previously determined [24,25].

2.3. Culture media and conditions

E. coli inocula were initially grown in LB medium for 8–12 h then transferred to M9 medium supplemented with succinate 20 mM and the appropriate surfactant under standing or stirring conditions at 30°C [26]. When needed the expression of the dioxygenase was activated by addition of 0.1 mM salicylic acid.

Viability experiments were performed by plating estimated amounts of cells, obtained after dilution with LB medium, on LB-agar plates. After 12–18 h incubation at 30°C, the grown colonies, corresponding to the viable cells, were manually counted.

Bioconversion experiments were performed at 30°C, 100–200 rpm shaking, adding to fresh M9 medium with the appropriate concentrations of surfactant, oil, naphthalene, and $3–40 \times 10^8$ cells/ml corresponding to about 10–120 U/l of naphthalene dioxygenase activity.

One unit of dioxygenase activity is defined as the amount of cells producing 1 μmol of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in 1 min at 30°C and pH 7.0.

2.4. HPLC determination of bioconversions kinetics

A reverse phase C18 column (4.6×250 mm) connected to a HPLC System Gold (Beckman, USA) comprising a 125 Solvent Module and a 168 Diode Array UV–Visible Detector interfaced to a personal computer was used under an isocratic flow (88–84% methanol, 12–16% water added with 1 g/l H_3PO_4) of 1 ml/min for the analysis of the reaction mixtures. 15 μl samples were injected after 3 min centrifugation at 14,000 rpm and 4°C. The absorbance of the eluate was monitored at 254 and 276 nm. The extinction coefficients for both the substrate (naphthalene) and the product ((+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene) were determined at both wavelengths and resulted not to change upon solvent change from pure water to M9/Tween 1% in water or in *n*-hexane. We therefore routinely used *n*-hexane solutions for calibration purposes because naphthalene is more easily dissolved in it than in water containing media. The absorption/concentration linearity for substrate and product in the concentration range utilized in the present work was also checked.

Known quantities of freshly prepared substrate or product solutions at established concentrations were injected in the HPLC system in order to allow the determination of unknown concentrations of both compounds at different times during the kinetic runs. A ratio of 1.7 between the absorbance of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene and that of naphthalene at 254 nm was observed. Under the conditions used during all the experiments a peak area (at 254 nm) of 1 for naphthalene corresponds to 5.3×10^{-5} M and a peak area of 1 for (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene corresponds to 3.1×10^{-5} M in the reaction mixture.

2.5. Phase diagrams

After the addition of a given amount of isopropyl-palmitate to the direct micellar solution (Tween 60 1% v/v or Tween 60 2% v/v in aqueous M9 medium), the samples were vigorously shaken for 2 min, then sealed and transferred into the water bath of a Haake Fisons C1 thermostat maintained at $30 \pm 0.1°$. A glass front window allowed direct observation of the phase behavior of each sample. The minimum equilibration time was 24 h.

2.6. UV–vis spectroscopy

UV measurements were carried out on double beam Perkin Elmer Lambda 5 or Varian

Cary 3 spectrophotometers using a 0.1 cm path length Hellma 110 quartz suprasil cells.

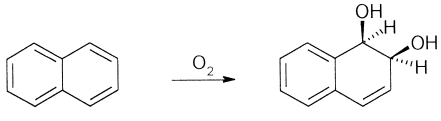
2.7. Light scattering

QELS experiments were carried out on a Brookhaven Instrument apparatus (BI 9000AT correlator card and BI 200 SM goniometer). The signal was detected by an EMI 9863B/350 photomultiplier. The light source was the doubled frequency of a Coherent Innova diode pumped Nd-YAG laser, ($\lambda = 532$ nm), linearly polarized in the vertical direction, whose power has been attenuated in order to avoid sample heating. The laser long term power stability was $\pm 0.5\%$. Self beating detection was recorded using decahydronaphthalene as index matching liquid.

The data were collected in multiple-sample time detection, and the autocorrelation function of the scattered light intensity was expanded about an average linewidth Γ as a polynomial function of the sample time with cumulants as parameters to be fitted, stopped to the second moment. A weighted least-square technique was applied to the second-order polynomial function to determine the constants and their standard deviation.

3. Results and discussion

The naphthalene dioxygenase subject of the present investigation catalyses reactions of the type:



giving rise to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene [24,25].

Aromatic *cis*-dihydrodiols have been recently shown to be valuable synthetic building blocks especially for highly stereoselective syntheses of biologically active molecules containing multiple chiral centers [18,40–42,26–31]. We have first determined the naphthalene solubility in M9 medium at 30°C from UV spectra, (naphthalene molar extinction coefficient $\varepsilon = 4923$ M^{-1} cm^{-1} at 277 nm, determined from a Beer-Lambert calibration in *n*-hexane, see Section 2) which resulted to be 3.8×10^{-5} M. This concentration does not allow optimal turnovers or the production of large quantities of dihydroxylated products in a homogeneous phase, therefore we attempted to increase the concentration of naphthalene in solution, and possibly its bioavailability. Suspensions of a naphthalene excess under vigorous shaking can also help in accomplishing such task but an enhanced solubilization of the substrate could increase its bioavailability and the reaction rates.

In several studies about whole cell solubilization in micellar systems the attention was focused on reverse micellar systems, where an enhanced solubilization of hydrophobic substrates could be easily accomplished [11].

Our first choice has then been addressed to reverse micellar systems, where a considerable gain in the degree of solubilization of the aromatic substrate is predictable, and in particular to Tween 85 (10% v/v)/IPP/LB (3% v/v) and Asolectin (10% v/v)/IPP/LB (1.5% v/v) media, since these non-ionic water in oil microemulsions have been previously used to dissolve bacterial cells in some pioneering studies [11] and a certain degree of viability has been shown to be retained [32]. The major drawbacks of L_2 phases concern however the viability of bacterial cells, and its determination, since the presence of an organic medium might interfere with usual Agar plating. Following the method proposed by Pfammatter et al. [10] after suspension of 106 cell/ml, we evaluated the viability of the *E. coli* strain, used for this study. No viable cells could be detected, even soon after the dissolution, thus indicating an immediate cell death, and this first approach had then to be discarded.

On the other hand, direct micellar systems can, in principle, increase hydrophobic substrate

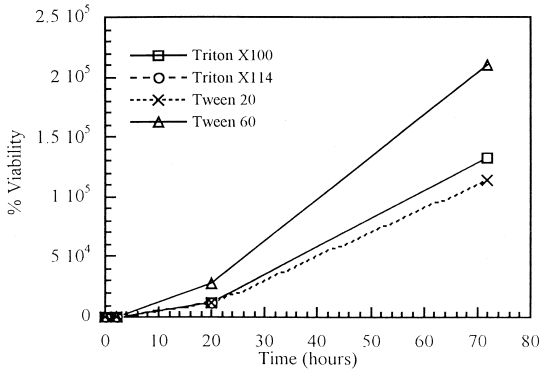


Fig. 1. Viabilities of the *E. coli* JM109(pPS1778) strain in the 1% v/v micellar systems tested under standing conditions.

solubility and provide a less aggressive environment for bacterial cells. It is known that ionic surfactants can easily damage cell membranes, while nonionic ones are milder in this respect. Therefore, we added 1–2% v/v of various nonionic surfactants of the polyoxyethylenesorbitan series (Tween 20, Tween 60), and of the alkylphenol–ethoxylate series (Triton X100 and Triton X114) to the culture medium (M9) for the *E. coli* strain under investigation. The viabilities of the strain in the modified media were tested in stirring (100 rpm) and standing conditions (see Fig. 1).

The Tween 60/M9 medium resulted to provide the best growth conditions among the systems tested, and our interest was then focused on this system.

Tween 60® (polyoxyethylene-(20)-sorbitan monostearate) is a nonionic surfactant, whose chemical structure is sketched in Fig. 2. It has been customary to sort nonionic surfactants according to a HLB (hydrophilic–lipophilic balance) number introduced by Shinoda [33]. This empirical scale allows to predict roughly the phase behavior of single and mixed surfactants and their emulsification properties. Nowadays an approach based on spontaneous curvature of the surfactant film is preferred [34]. The spontaneous curvature of the film depends on the chemical structure of the surfactant, but also on temperature, which affects the degree of hydration of the polar heads, on ionic strength and degree of oil penetration. Nevertheless, it is possible to compare those two approaches, considering a HLB close to 10 as the balanced state of spontaneous curvature ($H_0 = 0$), so that HLB numbers higher than 10 correspond to positive curvatures, which favours the formation of direct micelles or oil in water microemulsions, and HLB numbers lower than 10 mean a negative curvature, that is a spontaneous formation of reverse micellar phases or water in oil microemulsions.

The HLB number of Tween 60 is 14.9 and thus we are in the spontaneous positive curvature domain, so that direct micellar structures are to be expected; since the average molecular weight is about 1310 $gmol^{-1}$, and the density is 1.044 g/ml, a 1% v/v solution corresponds to a concentration of 8.0 10^{-3} mol/l and a 2% v/v to 1.6 10^{-2} mol/l. Both concentrations exceed the critical micellization concentration, which is around 27 mg/l.

The size and shape characterization of the 1% v/v and 2% v/v micellar systems has been obtained by quasi elastic light scattering spectroscopy, where local fluctuations of refraction index, due to the Brownian motions of the

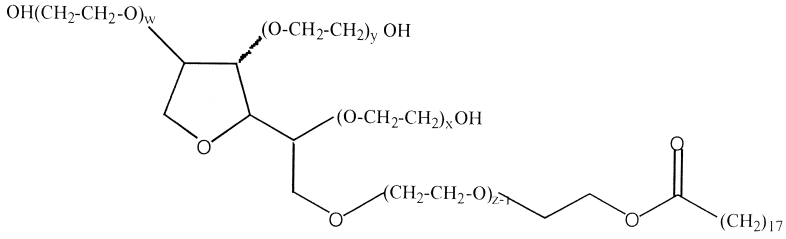


Fig. 2. Chemical structure of the Tween 60® (polyoxyethylene-(20)-sorbitan monostearate).

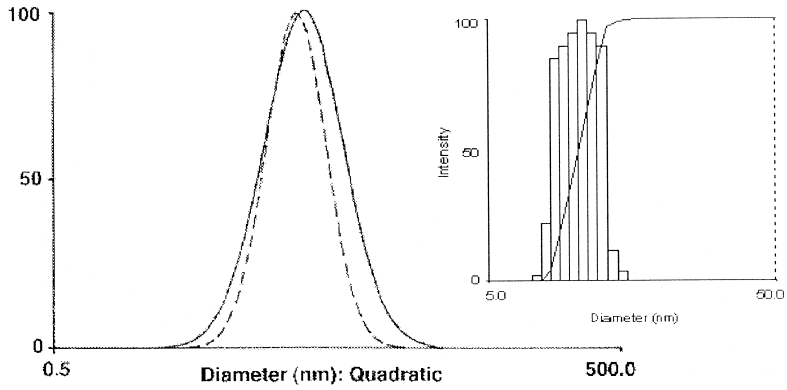


Fig. 3. Log-normal size distribution for 1% Tween 60 and 2% Tween 60 in M9 micellar solutions. A CONTIN histogram for the 1% system is reported in the inset.

particles in solution are collected and a scattered light intensity autocorrelation function is build up. *Z*-averaged diffusion coefficients can be inferred from the intensity autocorrelation function, and in the Debye–Stokes–Einstein approximation, they can be related to the hydrodynamic radius of spherical particles. However, since micellization is a thermodynamic equilibrium process, a micellar population is intrinsically polydisperse and thus, to interpret a light scattering experiment, some approximations have to be introduced for the data analysis. A very popular method, proposed by Koppel, is known as the cumulant method consisting in a polynomial expansion [35].

In Fig. 3, a log-normal size distribution, centered around the second-moment hydrodynamic radius, is reported for 1% Tween 60 and 2% Tween 60 in M9. The concentration increase results in a slight growth in average radius and polydispersity of the system. A CONTIN histogram for the 1% system reported in the inset, shows the size distribution of the micellar particles.

Micellar solubilization of apolar substances has been investigated by many authors and several models have been proposed [36,37]. Naphthalene has π-electrons which can be easily polarized and thus its solubility has to be expected higher in moderately polar solvents, such as lower *n*-alkanols. While the micellar interior is definitely apolar, and no water penetration is detected, the interface possesses dielectric properties similar to short-chain alcohols [38]. Therefore, a micellar system provides at least two differentiated compartments, with decreased average dielectric constant in comparison to bulk water, where a substrate could be located according to its polarity.

The addition of 1% Tween 60 to the M9 medium caused an increase of the solubility of naphthalene of two orders of magnitude with respect to water, reaching 3.8×10^{-3} M. No appreciable spectral shift of the low intensity solvent-sensitive bands of naphthalene (310–320 nm) could be detected with respect to *n*-hexane

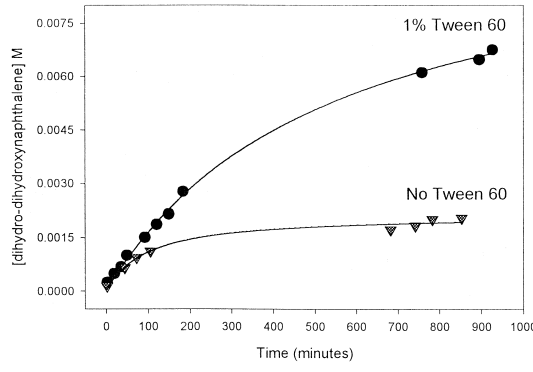


Fig. 4. Kinetics of production of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in suspensions of 0.1 M naphthalene in M9 and microemulsions of 1% v/v Tween 60 in M9. The final cell concentration of the induced *E. coli* JM109(pPS1778) recombinant strain resulted to be 6×10^8 cells/ml.

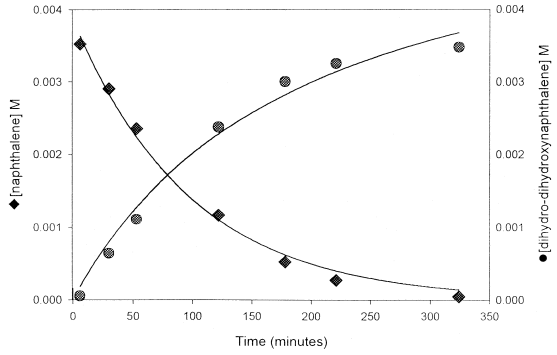


Fig. 5. Typical conversion kinetics in naphthalene saturated 1% v/v Tween 60 in M9 medium (initial naphthalene concentration 3.8×10^{-3} M). The final cell concentration of the induced *E. coli* JM109(pPS1778) recombinant strain resulted to be 8×10^{8} cells/ml.

and to saturated water, even if in the latter case a noisy baseline did not allow a rigorous comparison of such bands [39]. The apolar segment of Tween 60 is a stearate residue, which, in the micellar core, is known to have similar characteristics with respect to the alkane liquid state. Since naphthalene shows a scarce solubility in *n*-heptadecane or *n*-octadecane, a localization in the palisade layer is a reasonable hypothesis.

The kinetics of production of (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in suspensions of 0.1 M naphthalene in M9 and microemulsions of 1% Tween 60 in M9 are shown in Fig. 4 (cell concentration 6×10^{8} cells/ml). The presence of 1% Tween 60 greatly increases the conversion rates, probably enhancing the bioavailability of the hydrophobic substrate. The conversion in suspensions of 0.1 M naphthalene in M9 greatly decreases after 2–3 h probably due to cell death or enzyme inactivation. Therefore, the direct contact of naphthalene lumps with the cell membrane seems to interfere with the normal substrate uptake and cell metabolism.

Results from a typical conversion kinetics in naphthalene-saturated 1% Tween 60/M9 medium (final naphthalene 3.8×10^{-3} M) is reported in Fig. 5 (8×10^{8} cells/ml–21 U/l).

A further improvement for the microreactor would be achieved by the presence of oil droplets, separated from the aqueous medium by a surfactant film, where naphthalene could be very easily solubilized and stored before the conversion. A suitable apolar liquid has proved to be isopropyl-palmitate. Its moderate polarity ensures good solubility for naphthalene (exceeding 1 M). The *n*-alkane series, starting from *n*-decane on, fails to dissolve naphthalene to an acceptable extent. Lower *n*-alkanes, good solvents for naphthalene solubilization, cause immediate cell death, so that they had to be excluded.

Isopropyl-palmitate (IPP) was added to 1% v/v and 2% v/v Tween 60/M9 to a final volume ratio in the range 0.3–1.2%. The presence of a Winsor I phase, i.e., oil swollen micelles coexisting with an excess oil phase, was always observed in the temperature and composition ranges investigated. This result has been confirmed by dynamic light scattering data, as shown in Table 1. We observe no appreciable increase in the average micellar dimension, indicating that no progressive swelling is occurring, and that the swollen micellar phase remains unaffected by the amount of excess oil phase.

Table 1
Mean hydrodynamic radius and polydispersity index for Tween 60/M9 micellar solutions at different amounts of IPP containing 1 M naphthalene

IPP (% v/v)	Mean radius (nm) Tween 60/M9 1%	Poly index	Mean radius (nm) Tween 60/M9 2%	Poly index
0.0	9.6	14	10.4	25
0.3	10.9	16	10.8	20
0.6	10.5	15	10.6	18
1.0	10.2	16	10.2	17
1.2	11.4	25	11.0	18

According to the standard phase behavior of a nonionic surfactant, an increased surfactant concentration would presumably help, but a higher damage of bacterial cell membranes is also predictable, so that 1–2% v/v range appeared to be a good compromise. On the other side the addition of a cosurfactant, such as lower alkyl-chain alcohols (*n*-butanol or *n*-pentanol) proved to be cytotoxic.

An interesting parameter is the partition of naphthalene between the micellar phase and the excess oil phase. After 24 h of equilibration, the lower phase has been collected and its UV absorbance has been tested. The micellar concentration of naphthalene, determined from the absorption values, is reported in Table 2 for each sample, whereas the bulk value is shown in parenthesis. The substrate concentration in the micellar phase never exceeds the saturation values, since a partition in the oil phase is favoured. Changing from the 1% to the 2% v/v surfactant concentration, for a fixed amount of oil the ratio between naphthalene concentration values remains practically constant around 1.5.

The cell viability in these newly prepared systems was comparable to those previously observed.

The results of the HPLC determination of conversion kinetics with 6×10^8 cells/ml (16–17 U/l) final are shown in Fig. 6 for different quantities of IPP containing 1 M naphthalene (0.3, 0.6, 1.0, and 1.2% IPP v/v) added to 1% v/v Tween 60/M9 media. Such microemulsions resulted in apparent naphthalene concentrations of 3.0×10^{-3} (0.3% IPP) 6.0×10^{-3} (0.6% IPP) 1.0×10^{-2} (1.0% IPP) and 1.2×10^{-2} M (1.2% IPP). These data show the complete conversion of the substrate to the corresponding *cis*-dihydrodiol within 200–800 min depending on the naphthalene concentration. Using 1.2% IPP 1.54 g/l of product is easily obtained under the present experimental conditions. The initial conversion rates resulted to be comparable irrespectively of the IPP amount. Increasing the IPP reservoir (initially containing 1 M naphthalene) above the microemulsion maintains the environment saturated with naphthalene for longer times and therefore extends the productive reaction. Increases of the cellular concentration in the conversion assay up to 6×10^9 cells/ml resulted in a proportional in-

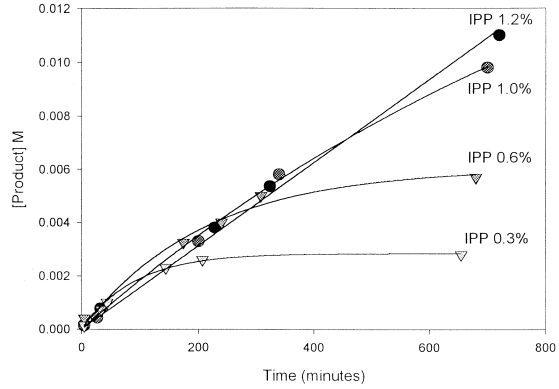


Fig. 6. Conversion kinetics in suspensions containing different amounts of isopropyl-palmitate (0.3, 0.6, 1.0, and 1.2% v/v, 1 M naphthalene) added to 1% v/v Tween 60 in M9 medium. The final cell concentration of the induced *E. coli* JM109(pPS1778) recombinant strain resulted to be 6×10^8 cells/ml.

Table 2
Naphthalene partition in the micellar phase for Tween 60/M9 1% and 2% at different IPP amounts containing 1 M naphthalene[a]

IPP (% v/v)	Naph (mol/l) in micellar phase (Tween 60 1%)	Naph (mol/l) in micellar phase (Tween 60 2%)
0.0	3.8×10^{-3}	5.1×10^{-3}
0.3	1.30×10^{-3} (3.0×10^{-3})	1.84×10^{-3} (3.0×10^{-3})
0.6	1.70×10^{-3} (6.0×10^{-3})	2.66×10^{-3} (6.0×10^{-3})
1.0	2.38×10^{-3} (1.0×10^{-2})	3.55×10^{-3} (1.0×10^{-2})
1.2	2.54×10^{-3} (1.2×10^{-2})	3.95×10^{-3} (1.2×10^{-2})

[a] In parenthesis are reported the bulk values of the naphthalene concentrations. The naphthalene concentrations reported for 0.0% IPP were determined after saturating the Tween/M9 media with such compound.

crease of the conversion rates thus suggesting that under the present experimental conditions the active enzyme concentration is the rate limiting step in substrate conversion.

4. Conclusions

The present investigation was aimed to the optimization of the biological transformation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene in microemulsions. This regio and stereoselective reaction is of enormous interest for asymmetric synthesis, since the product is a possible precursor for biologically active chiral compounds.

The low solubilities, and consequently the scarce bioavailability, of such compounds hinder efficient bioconversions.

We have devised a chemical bioreactor which allows to perform a quantitative conversion of large amounts naphthalene to its dihydroxylated derivative. Whereas aqueous systems with a naphthalene excess never result in a complete conversion of the aromatic substrates, whole cell bioconversion in direct micellar solution can yield a nearly complete transformation at higher rates. The addition of IPP, producing a reservoir for naphthalene, resulted in a further improvement in the bioconversion.

A challenging problem is now represented by a fast reagent–product separation, which could possibly be achieved through a phase separation induced by temperature or water/oil ratio changes. The optimization of the continuous and/or stepwise product separation is currently under investigation.

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