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Organic phase effect in the biphasic bioconversion of substituted naphthalenes by engineered *E. coli* containing *P. fluorescens* N3 dioxygenase

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Abstract

Recombinant *Escherichia coli* based on *Pseudomonas fluorescens* N3 naphthalene dioxygenase (NDO) was used in two liquid phase bioconversions to produce 1,2-dihydro-1,2-dihydroxy naphthalenes derived from substituted naphthalenes. Five different organic phases (dodecane, silicon oil CR100, dioctyl phthalate, 1-dodecanol, 2-undecanone) were used at different ratios with respect to water and containing different substrate concentrations. The results show different phase effects in terms of both rates and other experimental variables. The differences between substrates are very interesting, showing rate increases from 0.9 to 4 times. The choice of the phase to some extent depends on the substrate, but dodecane shows to be a general effective phase for all the substrates.

Keywords: Naphthalene dioxygenase; Biphasic system; Bioconversion; Whole cell biocatalyst; Substituted naphthalenes

1. Introduction

Since many years our group is interested in the bioconversion of naphthalenes into enantiopure 1,2-dihydro-1,2-dihydroxy derivatives by the dioxygenase isolated from *Pseudomonas fluorescens* N3 strain. This enzyme activity stereospecifically introduces two hydroxyl groups onto the aromatic ring of naphthalenes, showing good transformation yield for many substrates. Due to the straightforward application, in our case, of whole cell bioconversion in comparison to the complexity of enzyme isolation and purification, we chose that system as our standard procedure. However, when we passed to a larger scale production of 1,2-dihydro-1,2-dihydroxy naphthalenes we were forced to consider some new aspects that were specific of the new development. One of these is the necessity to have a stable and efficient system for substrate delivery. The use of a second, organic, phase can resolve the problem contributing to the regular delivery of the substrate to the cell. However, it can change the transport mechanism, or even affect the cell functioning. In this respect, the chosen solvent, its ability to dissolve the substrate, and consequently the substrate concentration, and the ratio between the organic and the water phases, are all important factors that can be strongly influenced by the solvent–substrate pair [1–11]. In this work we investigated the organic phase effect in the 1,2-dihydro-1,2-dihydroxy naphthalenes production from substituted naphthalenes.

2. Experimental

2.1. Materials and methods

2.1.1. Biocatalyst growth conditions in flask

E. coli JM109 (pVL1343A + pMS13) [12] is maintained in glycerol solution (40%, w/w) at −80 °C. When needed it is grown on a plate containing LB (Luria–Bertani)

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[13] medium, solidified with 1.5% agar, containing ampicillin, kanamycin and salicylic acid (final concentrations: 100 μg/mL, 50 μg/mL and 1 mM, respectively) at 30 °C for 2 days. A single blue colony is then grown overnight at 30 °C in a 100 mL flask containing 20 mL of LB solution, containing the corresponding amounts of antibiotics and inducer.

Finally, 10 mL of the culture are centrifuged (10 000 rpm, 4 °C, 10 min), cell collected, and added to 100 mL of M9 [13] medium, in a 500 mL flask, containing ampicillin, kanamycin, and salicylic acid in the usual concentrations; glucose (0.2%, w/v) and thiamine (0.05 mM) are added; the culture is grown overnight at 30 °C.

2.1.2. Biocatalyst growth conditions in 3 l bioreactor

All the previous operations are also used to prepare the starting cells when the final growth is performed in a 3 l bioreactor. The final growth is performed using the cells coming from a standard flask growth. Hundred milliliters of the culture are added to 900 mL of a chemically defined medium (Wang-Lee) [14], containing glucose (20 g/l), thiamine (1 mM), antibiotics and inducer at the usual concentration. The growth is performed overnight at 30 °C. The cells are harvested by centrifugation (10 000 rpm, 4 °C, 10 min) and stored at −20 °C.

2.1.3. Bioconversion cultures

When needed the required amount of cells is unfrozen by shaking at 30 °C for 1 h in M9 medium containing glucose. Then, the culture cell density is adjusted at the required value by addition of M9 medium; finally, glucose (initial concentration 0.2% (w/v); similar amounts added when needed) and the substrate are added and the transformation is performed in a flask (usually 250 mL containing 50 mL of culture) at 30 °C on a horizontal shaker.

In both biocatalyst production and bioconversion the culture media are sterilized at 131 °C, 10 min; or by filtration on 45 μm Millipore filters; all equipments are autoclaved.

Cell density is measured using a Shimadzu photometer at 600 nm. Glucose is monitored using enzyme sticks (Glukur Test) from Roche.

2.1.4. Bioconversion analysis

Diol production is monitored analysing the water phase by HPLC, Hitachi-Merck, UV-Vis detector at 254 nm, reversed phase column C18 (Hibar LICHROSORB 50334, 10 μm, 25 cm), H_2O:CH_3CN 1:1 eluent, 1 mL/min flow, Hitachi D2500 integrator.

The naphthalene and diol amount in the organic phases is measured using GLC analysis. GLC Dani 1000, capillary column CP-sil 8CB (CHROMPACK), FID detector (FID-861), using the following conditions: $T_{injector}$, 150 °C; $T_{detector}$, 300 °C; T_{column}, at 105 °C for 5 min, then 10 °C/min rate to 250 °C; carrier gas, helium; P_{He}: 0.8 bar; internal standard: 2-methoxynaphthalene or naphthalene. Data variance is mainly connected to the precision of the instruments used in the analysis; it is thus possible to assume a ±5% deviation.

^{1}H NMR spectra are obtained in $CDCl_3$ or CD_3OD (Merck) using Bruker AC-300 and Bruker AC-200 instruments.

2.1.5. Bioconversion comparison

To make a comparison between different experiments we prepared reference bioconversions for each series. A first standard bioconversion is performed using solid naphthalene (3 g/l) as substrate in a reference flask containing exactly the same culture (same cells and same density) in M9 mineral medium, containing glucose (initial concentration 0.2%, w/v). Both substrate and glucose are added when necessary to exclude their influence on the conversion rate. This value is used to evaluate the biocatalyst activity. This has two results: first, we obtain a measure of the current cell activity; second, we guarantee the reliability of the current experiments. A second standard bioconversion is performed using the current pure substituted naphthalene (3 g/l) as substrate in the same conditions; this last is used to calculate the specific activity ratio reported in the tables. Thus, it is always possible to calculate absolute values characteristics of the current conditions and independent of the current biocatalyst.

Each bioconversion rate is expressed as $mmol_{diol}\ h^{-1}\ l^{-1}\ DCW^{-1}$ and is corrected by the diol present in the organic phase. The rate ratio is a pure number and it is calculated dividing the activity of the current system containing the solvent and the reference experiment performed using the same substrate in water. When using 1-nitro naphthalene as substrate the measure of the activity of the substrate in water was impossible because the bioconversion stopped immediately, therefore we used the naphthalene bioconversion as reference.

2.2. Chemicals

All the chemicals are from Acros, Sigma Aldrich, Oxoid, or Fluka. The HPLC solvents are from LAB-SCAN. TLC plates (Alugram SL G/UV254) are from Merck.

3. Results and discussion

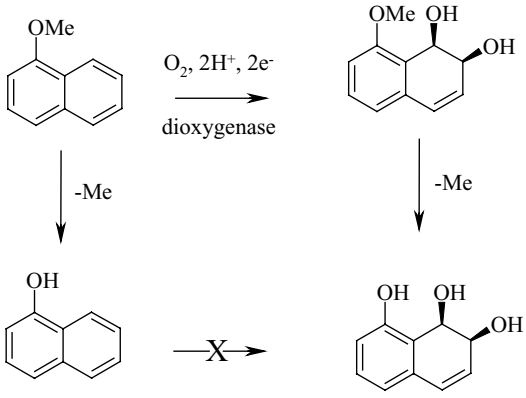
We have studied the bioconversions by *E. coli* JM109 (pVL1343A + pMS13) of several substituted naphthalenes and of two tricyclic compounds. All the compounds are solid excluding 1-methoxy, 1-carbomethoxy, and 1-bromo, naphthalenes. The selected organic phases were: dodecane, silicon oil CR100, 2-ethyl hexyl (dioctyl) phthalate, 1-dodecanol, and 2-undecanone. In the following we will report only some of the obtained results, selecting those which have a special meaning; the complete set of results, including negative ones, is available from the authors.

Table 1
Substrate concentration at phase saturation[a]

Substrate	Solvent	Concentration (g/l–mol/l)
Naphthalene	Dodecane	90–0.7
	1-Dodecanol	75–0.59
	2-Undecanone	160–1.25
	Dodecane/1-dodecanol 1/1	80–0.63
	Dodecane/2-undecanone 1/1	140–1.09
	Silicon oil CR100	20–0.16
2-Carbomethoxy naphthalene	Dodecane	45–0.24
	1-Dodecanol	40–0.22
	2-Undecanone	150–0.84
	Dodecane/1-dodecanol 1/1	40–0.22
	Dodecane/2-undecanone 1/1	75–0.4
	Dioctyl phthalate	50–0.27
2-Methoxy naphthalene	Dodecane	50–0.32
	Dioctyl phthalate	135–0.85
	1-Dodecanol	60–0.38
2-Bromo naphthalene	Dodecane	160–0.77
	Dioctyl phthalate	55–0.27
	1-Dodecanol	30–0.14
1-Carbomethoxy naphthalene	All solvents	>100–>0.54
1-Methoxy naphthalene	All solvents	>100–>0.63
1-Bromo naphthalene	All solvents	>100–>0.48
1-Nitro naphthalene	Dodecane	35–0.2
	1-Dodecanol	10–0.06
	2-Undecanone	180–1.04
	Dodecane/1-dodecanol 1/1	15–0.09
	Dodecane/2-undecanone 1/1	65–0.38
	Dioctyl phthalate	45–0.26
	Silicon oil CR100	5–0.03
Anthracene	Dodecane	45–0.25
	Dioctyl phthalate	25–0.14
	1-Dodecanol	15–0.08
Phenanthrene	Dodecane	55–0.31
	Dioctyl phthalate	35–0.2
	1-Dodecanol	25–0.14

[a] Substrate amounts were measured after addition of enough substrate to have part of it undissolved and leaving under stirring for 2 h at room temperature. Values do not appreciably change in the 2 h.

The solubility of the substrates into each solvent varies quite widely; the saturation concentration are reported in Table 1. In addition, some data concerning the partitioning of naphthalene between water and organic phases have been measured. Partitioning with respect to solid naphthalene in water (set at 1) are: dodecane 1.8, dioctyl phthalate 1.2, 1-dodecanol 1.7, and 2-undecanone 1.2. Three compounds (naphthalene, 2-carbomethoxy naphthalene, 1-nitro naphthalene) were analyzed in greater detail; they ideally represent three classes of compounds: the reference substrate, 2-substituted substrates, 1-substituted substrates. In addition, in experiments performed without a second phase they also show different relative transformation rates (100, 20, 5, respectively) [12]. In these cases, we tested four or five solvents and at least two solvent mixtures. The remaining substrates were tested using only those solvents that demonstrated more interesting (dodecane, dioctyl phthalate, and 1-dodecanol). 1-Methoxy naphthalene was not analyzed in all the solvents; in fact when used as substrate the product is not the usual diol but the demethylated analogue (Scheme 1). The demethylation is probably due to an *E. coli* activity and forbids the use of this compound because the resulting 1-naphthol is toxic to the microorganism and is not transformed by the enzyme (Table 2).



Scheme 1. Demethylation and dioxygenation of 1-methoxy naphthalene. 1-Naphthol is not transformed by the enzyme.

Table 2
Naphthalene, 2-carbomethoxy naphthalene, and 1-nitro naphthalene

Entry	Substrate	Conditions	Substrate concentration[a] (g/l–mol/l)	Reference activity[b] (mmol h^{-} DCW^{-1} l^{-1})	Specific activity ratio[c]
1	Naphthalene	8/2 dodecane	30–0.23	1.97	1.47
2		8/2 dodecane	80–0.63	2.54	1.48
3		7/3 dodecane	50–0.39	4.93	1.28
4		7/3 phthalate	50–0.39	4.93	1.53
5		8/2 silicon oil CR100	15–0.12	6.87	1.25
6		8/2 1-dodecanol	70–0.55	2.54	0.48
7		8/2 2-undecanone	150–1.17	2.54	0.25
	2-Carbomethoxy naphthalene	No solvent[d]		3.42	0.2
8		9/1 dodecane	10–0.054	2.06	0.57
9		9/1 dodecane	25–0.13	4.05	0.73
10		9/1 dodecane	30–0.16	2.06	0.83
11		9/1 dodecane	40–0.22	3.11	0.83
12		8/2 dodecane	30–0.16	1.65	0.83
13		7/3 dodecane	10–0.054	2.06	0.70
14		7/3 dodecane	30–0.16	2.06	0.87
15		1/1 dodecane	25–0.13	4.05	0.92
16		9/1 1-dodecanol	35–0.19	3.71	0.05
17		9/1 2-undecanone	10–0.05	3.15	–
	1-Nitro naphthalene	No solvent[d]		3.96	0.04
18		9/1 dodecane	10–0.058	1.72	0.17[e]
19		8/2 dodecane	30–0.17	6.69	0.04[e]
20		7/3 dodecane	10–0.058	1.72	0.14[e]
21		1/1 dodecane	10–0.058	1.72	0.12[e]
22		9/1 dioctyl phthalate	10–0.058	4.37	0.1[e]
23		9/1 dioctyl phthalate	30–0.17	5.33	0.15[e]
24		7/3 dioctyl phthalate	10–0.058	4.37	0.12[e]
25		7/3 dioctyl phthalate	30–0.17	5.33	0.11[e]
26		1/1 dioctyl phthalate	10–0.058	4.37	0.08[e]
27		1/1 dioctyl phthalate	30–0.17	5.33	0.11[e]
28		8/2 1-dodecanol	10–0.058	6.96	0.06[e]
29		8/2 2-undecanone	170–0.98	6.96	0.03[e]

Bioconversion experiments in biphasic solvents.
[a] Initial substrate concentration in the organic phase.
[b] Biocatalyst activity measured using naphthalene as substrate dispersed in water medium.
[c] Activity ratio with the reference experiment performed using the substrate dispersed in the water medium.
[d] Average activity of the current compound compared to naphthalene.
[e] Activity ratio with the reference experiment performed using naphthalene, and not 1-nitro naphthalene, dispersed in the water medium.

3.1. Naphthalene, 2-carbomethoxy naphthalene, 1-nitro naphthalene

Naphthalene was already subjected to preceding experiments [15,16], thus only some key conditions and some new solvents have been tested. Dodecane and dioctyl phthalate confirm their value increasing by ∼50% the rate. In dodecane, we cannot observe any effect of the substrate concentration (entries 1 and 2), in agreement with previous data, and any phase ratio effect (entries 2 and 3). All other solvents give negative results.

Solvent effects are much more visible in the case of 2-carbomethoxy naphthalene. Here, we can observe both a substrate concentration and a phase ratio effect. In dodecane, we observe a rate increase when the substrate concentration is high (entries 8–11); in addition, a similar effect on the rate is observed at high phase ratio maintaining constant the substrate concentration (entries 10, 12, 14, and 15).

In the absence of an organic solvent the bioconversion rate of 1-nitro naphthalene is so low that it is hard even to register the experiment. Its usual transformation yield is 5% of that of naphthalene and the conversion stops after very short time (30–60 min). This result is so negative that, contrary to all the other cases, we reported the rate ratio using naphthalene as the reference. The use of all solvents causes two positive consequences: the transformation continues for longer time and the rate becomes appreciable. Clearly, the comparison must, in this case, take into account that naphthalene is the natural substrate for the enzyme and, consequently, it is highly favored when the activity is high. In dodecane there is no clear phase ratio effect (entries 18, 20, 21); also the substrate concentration seems unimportant (entries 18 and 19) even if this single comparison is not conclusive. When using phthalate the situation changes; the rates are constantly superior than in dodecane; the substrate concentration favors the reaction, whilst the phase ratio is less important. In these conditions 1-nitro naphthalene is

Table 3
Other substituted naphthalenes and tricyclic compounds

Entry	Substrate	Conditions	Substrate concentration[a] (g/l–mmol/l)	Reference activity[b] (mmol h^{-1} DCW^{-1} l^{-1})	Specific activity ratio[c]
	2-Bromo naphthalene	No solvent[d]		5.32	0.12
30		9/1 dodecane	160–0.77		
	2-Methoxy naphthalene	No solvent[d]		3.5	0.37
31		9/1 dodecane	45–0.28		
32		9/1 dioctyl phthalate	13–0.82		
	1-Carboxy-naphthalene	No solvent[d]		5.31	0.22
33		9/1 dodecane	50–0.27	1.14	1.17
34		9/1 dodecane	100–0.54		
	1-Bromo naphthalene	No solvent[d]		5.93	0.14
35		9/1 dodecane	50–0.24	5.20	1.24
36		9/1 dodecane	100–0.48	6.65	1.63
	Anthracene	No solvent[d]		2.32	0.01
37		9/1 dodecane	40–0.022	2.32	1.46
	Phenanthrene	No solvent[d]		3.38	0.07
38		9/1 dodecane	45–0.025	3.38	0.99

Bioconversion experiments in biphasic solvents.
[a] Initial substrate concentration in the organic phase.
[b] Biocatalyst activity measured using naphthalene as substrate dispersed in water medium.
[c] Activity ratio with the reference experiment performed using the substrate dispersed in the water medium.
[d] Average activity of the current compound compared to naphthalene.

oxidized as well as 2-carbomethoxy naphthalene and the other 1-substituted compounds. Contrary to all the other experiments 1-dodecanol and 2-undecanone (entries 28 and 29) also improve the rate, confirming that the bioconversion is greatly hindered in the solvent absence.

3.2. Other substituted naphthalenes and tricyclic arenes

2-Bromo and 2-methoxy naphthalenes were expected to give results in line with naphthalene and 2-carbomethoxy naphthalene, respectively; however, the results were partly different. 2-Bromo naphthalene is much more soluble in dodecane than naphthalene and, as a consequence, its conversion rate greatly improves. In addition, the use of the other solvents gives negative results. Interestingly, the solubility of 2-methoxy naphthalene in the different solvents is reversed compared to that of 2-bromo naphthalene, but the rate are not affected in the same direction. 2-Methoxy naphthalene has rates comparable in dodecane and phthalate (entries 31 and 32), despite the difference in solubility (Table 3).

1-Carbomethoxy and 1-bromo naphthalenes are the only two liquid compounds; they easily dissolve in organic solvents. We used two substrate concentrations (50 and 100 g/l) as representative concentrations. 1-Bromo naphthalene gives results in agreement with its 2-substituted analogue: good yields in dodecane, increasing with concentration, low yields in the other solvents. 1-Carbomethoxy derivative also prefers dodecane as its 2-substituted analogue, but it shows a reverse sensitivity to concentration: the greater this last, the lower the rate.

Anthracene and phenanthrene are pure hydrocarbons and they give results in good agreement with naphthalene. Dodecane is always the best solvent and its use guarantees an increase in the conversion rate. Anthracene remains the worst substrate for the enzyme because of its shape, as already known [17].

3.3. Solvent choice

It is worth to remember that the purpose of the present work is the selection of an organic phase that is essential to perform bioconversions of naphthalenes at bioreactor level; the goal is thus the selection of the best phase. From the results, it is clear that different substrates show different behaviors when the bioconversion is carried out in the presence of a second phase. With respect to the water phase experiments, using a second phase: there are substrates that show a rate enhancement (naphthalene, 2-bromo, 1-nitro, 1-carbomethoxy, 1-bromo, naphthalenes, and anthracene); others that show a rate of the same extent (2-methoxy naphthalene and phenanthrene); 2-carbomethoxy naphthalene that reacts at a slower rate.

A complete rationalization of the results is difficult, but we can consider some experimental indications. First, all the substrates are hydrophobic and they are easily dissolved in organic solvents. Second, the cell membranes is also lipophilic and it has been demonstrated that the cells have a direct interaction with the organic phases. Third, it seems that the bioconversion does not occur exclusively on the substrate dissolved in the water phase. Fourth, we noted that the substrate transfer rate can be a determining factor of the bioconversion efficiency, as shown by the dependence on the stirring mode and speed [15]; however, at low biocatalyst activity the substrate availability is no more a limiting factor. Taking into account all these indications we can formulate a hypothesis concerning the solvents used in the present work:

dodecane (or other hydrocarbons) is the only solvent that has a direct interaction with the cell, enhancing the substrate transfer. As a consequence, we can expect a transformation rate enhancement in dodecane: if the substrate is highly soluble; if the substrate concentration is high; if the phase ratio is high. In addition, because we compare the reaction efficiency to that occurring in water, we must remember that the partitioning rate between two liquid phases is lower than the partitioning rate between the pure compound and the water.

In this perspective, all the non-oxygenated compounds show an appreciable rate enhancement using dodecane; on the contrary, the oxygenated compounds are scarcely affected by the solvent presence, unless their transformation in water is very low (e.g. 1-nitro naphthalene). This is confirmed by

1. The substrate concentration in the organic phase has the best effect at phase saturation. In this sense, 2-bromo naphthalene is enlightening; in fact, because its solubility in dodecane is very high (160 g/l) the reaction rate shows a notable 200% increase.
2. The phase ratio effect is less evident; in most of the cases the rate is not significantly affected.

There are some other considerations that help in the solvent selection. Other things being equal: the solvent that does not extract the product from water permits an easier product recovery; it is preferable to operate at phase ratios in favor of water because this gives a greater absolute yield.

In conclusion, we can suggest dodecane as the preferred solvent for all the substrates that do not contain many polar groups, whilst dioctyl phthalate can become the first choice in the presence of polar groups (e.g. 1-nitro naphthalene). In addition, we can expect a rate increase only when the substrate availability is one of the limiting factors of the bioconversion. Nevertheless, a scaled-up bioconversion can be planned with all the substrates in a biphasic environment, thus assuring the constant substrate presence during the reaction.

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