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Bioconversion of Substituted Naphthalenes to the Corresponding 1,2-Dihydroxy Derivatives by *Escherichia coli* Recombinant Strains

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Abstract: 1,2-dihydroxynaphthalenes are produced by bioconversion of the corresponding hydrocarbons using *Escherichia coli* recombinant strains containing the naphthalene dioxygenase and dehydrogenase genes cloned from *Pseudomonas fluorescens* N3. Conversions are lead by a two step procedure without isolation of the dihydrodiol intermediate. Conversion rates depend on the position and nature of the naphthalene substituent © 1997 Elsevier Science Ltd.

The use of microbial bioconversions to synthesise chemical compounds is becoming an everyday alternative to classical chemical synthesis. The specificity and the regio- and stereoselectivity of the enzymatic transformations combined with the mild reaction conditions and the use of inexpensive reagents represent sound advantages that must be considered each time a transformation can be realised microbiologically. Moreover the continuous development of genetic methodologies has permitted the achievement of controlled and highly productive engineered microrganisms.

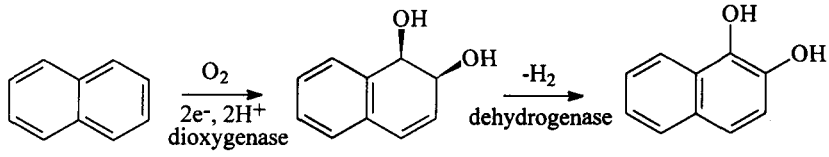
The utility of 1,2-naphthalenediols and of the corresponding quinones is well known. The diols are often used as copolymer components.[1] 1,2-naphthalene diol itself shows an emetic and anti-Parkinsonism effect.[2] The 1,2-quinones are much more studied as pharmacologically active substances (e.g. fungicides,[3] cytotoxic and antitumoral compounds[4]). On the other hand, their preparation methods are relatively scarce. In particular, the diols are often derived from the quinones by reduction.[5] The quinones can be obtained: a) from the benzene analogues by a synthetic sequence involving initial condensation with pyruvic acid and requiring forcing conditions;[3] b) from β-naphthols by oxidation in particular basic conditions.[6] The yields are quite often scarce (10-30%), therefore the objective of their production by bioconversion is appealing.

Using this methodology we have already realised bioconversion systems for the production of optically pure 1,2-dihydro-1,2-dihydroxynaphthalenes in high yield[7] and, adding a pure chemical step, of 1- and 2-

naphthols.[8] This result has been reached after the isolation and characterisation of a *Pseudomonas fluorescens* N3 able to grow using naphthalene as the sole energy and carbon source and carrying the plasmid pN3 responsible for the naphthalene degradation. The isolation and cloning of the naphthalene dioxygenase gene into an *E. coli* JM109 recipient (leading to a recombinant strain JM109(pPS1778))[9] gave us the desired microbial system. The catabolic operons are under positive control by many intermediates of the degradative pathway of naphthalene (naphthalene itself and salicylic acid have proved their activity).[10] This microrganism can transform naphthalene, many 1- and 2-substituted naphthalenes, and some other aromatic compounds (e.g. phenanthrene, anthracene, fluorene, *cis*-stilbene) into the corresponding 1,2-dihydro-1,2-dihydroxy derivatives in yields ranging from 1 g/L to several g/L. The obtained diols are surprisingly stable and eliminate water, giving the corresponding aromatic compounds, only when treated with acids or warmed.[11] Continuing our analysis of the *P. fluorescens* N3 metabolism we decided to localise and engineer the degradative step involving the naphthalene 1,2-dihydrodiol dehydrogenase with the goal of producing 1,2-dihydroxy naphthalenes.

The 2.8 kb BamHI fragment carrying the dehydrogenase has been identified and cloned in the pVLT33 vector[12] leading to the pVL2028 plasmid introduced in *E.coli* JM109. The recombinant strain JM109(pVL2028) showed the ability of converting 1,2-dihydro-1,2-dihydroxy naphthalenes into the corresponding 1,2-dihydroxy derivatives. Having the microrganism available, we began the search for a transformation system that could efficiently use the strain to operate the bioconversion.

The main problem when producing 1,2-dihydroxy arenes is represented by their easy oxidation to the corresponding quinones, first, and by the subsequent polymerisation. In fact, carrying the transformation in the usual bioconversion conditions (30 °C, air, water, 1g/L concentration) the only result is the very fast formation of a black tar made by polymerised naphthalene catechol by-products. However, it is known that *E.coli* is an anaerobic facultative microrganism, able to grow in the absence of oxygen. As the reaction we are interested in does not require the presence of oxygen, being a dehydrogenation reaction, it is possible to conceive a system working in inert atmosphere (under nitrogen). There still remains different possibilities of conceiving the transformation system. The desired result is the production of 1,2-dihydroxy naphthalenes in good conversion yield, in reproducible conditions, with good product recovering, and, last but not least, with the smallest number of operations.



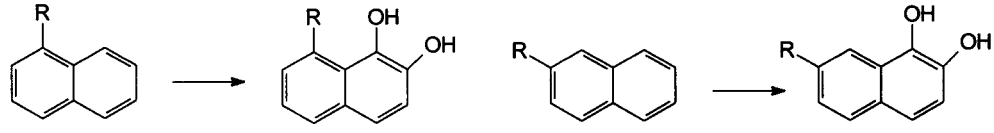
Scheme 1

Two step oxidation of naphthalene: dioxygenation and dehydrogenation

The system we realised uses at its best all the features of the considered bioconversions.[13] It is made of the following steps: the production of the diol is obtained in the usual conditions[9] but operating at doubled cell and substrate concentrations[14] (O.D. equal to 2; 2 g/L); then the solution is centrifuged, diluted with an equal

volume of fresh medium, accurately deareated with nitrogen, and finally added with JM109(pVL2028) (at final O.D. equal to 1). After the conversion time the solution is extracted with ethyl acetate under a nitrogen atmosphere. The 1,2-dihydroxy naphthalenes are sufficiently stable in organic solvents to be isolated and characterised.

With this system we transformed several naphthalenes into the corresponding 1,2-dihydroxy derivatives with success (Figure 1). In Figure 2 the curves relative to the conversion of each substrate in the described conditions (the curves describe the rate of diol consumption)[15] are reported and deserve few comments.



R = C_2H_5, Br, Cl, COOMe, H

R = C_2H_5, Br, Cl, COOMe, Me, OMe

Figure 1

1,2-dihydroxy naphthalenes obtained by bioconversion

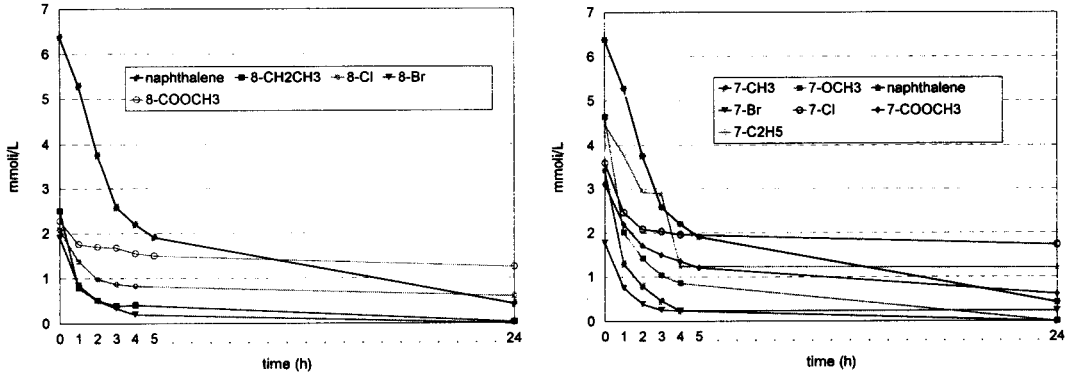


Figure 2

Rate of conversion of 1,2-dihydro-1,2-dihydroxynaphthalenes into corresponding 1,2-dihydroxy derivatives

First, the overall yield depends on the initial concentration of the diols; second, the conversion rate depends on the nature and the position of the substituent. Concerning the first aspect, it is clear that the greater is the amount of the diol to be transformed the more we must optimise the system to get its complete conversion. In fact, we are already working in that direction, e.g. changing the concentration and the nature of the cosubstrate used to renew the NAD^+ cofactor needed for the oxidation. Concerning the second aspect, we are going to carry some specific transformations at fixed initial diol concentration and at measured enzyme activity. It can be note that the conversion has proceeded to 35-90 % in the first four hours.[16]

In conclusion we built a bio-system capable of transforming naphthalene and some of its derivatives into the corresponding 1,2-dihydroxy derivatives. To our knowledge, this is the first reported system that uses

microrganisms and not isolated enzymes,[17] and that permits the isolation of the products in overall good yield. We are working to further optimise the bioconversion, both changing the system conditions and developing new engineered strains.

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11. There are, however, some exceptions: for example one of the two regioisomers derived from *cis*-stilbene or the diols of fluorene.
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13. Ideally the transformation could be imagined as occurring in one pot, i.e. mixing in the same system the microrganisms carrying the two activities and starting the two enzyme productions at successive time. This could have been possible because JM109(pPS1778) is inducible by salicylate, whereas JM109(pVL2028) is inducible by IPTG (IsoPropyl-β-D-ThioGalactopyranoside). Consequently we could begin the second transformation when the first is already finished and in the absence of oxygen, that is absolutely needed in the first conversion (Scheme 1). Unfortunately the level of basal dehydrogenase activity is sufficient to produce enough enzyme to destroy (in the air) all the final product. On the opposite side there is the possibility of making the two transformations separately with the isolation of the first intermediate (1,2-dihydro-1,2-dihydroxy diol). This solution would clearly work, but the number of operations together with the need of extracting the compound, i.e. of using solvents, etc., makes it unattractive.
14. Because the solution coming from the first conversion is diluted, we would like to have a higher concentration of diols.
15. It is impossible to follow the increase in 1,2-naphthalene diol concentration because its oxidation in air is too fast to give reliable data. Consequently we used the rate of dihydrodiol disappearance that has been monitored by HPLC: Merck-Hitachi instrument; RP-8, 250 mm, 5μ, spherical, column; CH_3CN/H_2O 50/50 eluent, 1 mL/min flow rate; samples were collected each hour for five hour periods and after 24 h.; sample areas and the corresponding diol amounts were calculated using calibration lines.
16. All products have been isolated and identified by ^{1}HNMR spectroscopy. The yield of the isolated catechols are in agreement with the conversion of the corresponding diols as determined by HPLC. The spectra do not show any trace of compounds besides the catechols and the uncoverted diols.
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