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Choice of microbial host for the naphthalene dioxygenase bioconversion

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The use of whole cell biotransformations for single and multistep enzyme conversions is gaining widespread application. In this study the naphthalene dioxygenase *nah* A gene was transferred into *Pseudomonas aeruginosa* PAC 1R, *Escherichia coli* JM107 and *Pseudomonas putida* PpG 277. The effect of ethanol on these genetically engineered Gram-negative bacteria was studied by measurement of enzyme activity, stability and cell integrity. Ethanol has been used in biotransformations as a co-substrate carbon source for co-factor recycling and as a co-solvent increasing dissolved substrate and product levels. Ethanol increased the dissolved substrate (naphthalene) concentration slightly and dissolved product ((+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene) by approximately 30% at 4% (w/v) ethanol. Both *P. aeruginosa* PAC 1R and *P. putida* PpG 277 showed decreased activity with increasing ethanol concentration whilst *E. coli* enzyme activity increased with increasing ethanol concentration being comparable to that when glucose was used as a carbon source. This project highlighted the many factors involved in the selection of microbial hosts for whole cell biotransformation processes.

Keywords: bioconversion; ethanol; naphthalene dioxygenase; co-solvent; microbial host

Introduction

Microbial biocatalysts and isolated enzymes enable a range of difficult chemical reactions to be undertaken often at ambient temperature, atmospheric pressure and near neutral pH in single catalytic steps [10,25,38]. In addition, many enzymes show enantioselectivity and regiospecificity enabling the production of optically pure compounds [44,45]. The use of an intact cell as the biocatalyst is necessary in those reactions where isolation of the required enzymic activity is not possible or those that require expensive cofactors to be recycled [7]. However the conversion of hydrophobic substrates under the aqueous conditions required by intact microbial biocatalysts has been limited to a large extent by low substrate and product solubilities. Feeding strategies [16] can be used to overcome substrate solubility limitations but may lead eventually to product inhibition [12]. Alternatively the addition of organic solvents to the biotransformation medium either in subsaturating concentrations [8] or in excess of saturation to form a second liquid phase [30,31,47] has been widely used as a method to overcome these limitations. Whilst the addition of an organic solvent to an aqueous biotransformation medium may overcome the difficulties of substrate and product solubility (and/or inhibition/toxicity), it may also have detrimental effects on the biocatalyst. Recently therefore a number of studies have addressed the need for a parameter to correlate the potential detrimental effects of a solvent on a particular biocatalyst.

The most widely accepted physical parameter to indicate solvent toxicity is that of solvent Log P [27,28] (where P is the partition coefficient of the solvent between octanol and water [36]). A sigmoidal relationship exists between enzyme activity and Log P, with those solvents having Log P values >3 (ie the more hydrophobic solvents) being the most biocompatible [27,28]. The amounts of primary alcohol required to inhibit the 11α hydroxylase activity in *Rhizopus nigricans* [35] and inhibit microbial growth [17] decreased with increasing chain length, seemingly contrary to the idea of higher log P solvents being most biocompatible. This suggested that at subsaturation concentrations, the metabolisable primary alcohol, ethanol (Log P = 0.24) may have some use both as a carbon source and a co-solvent. Ethanol has been successfully used previously at subsaturation levels (2% w/v) as a carbon source/co-substrate in the conversion of toluene to toluene-*cis*-glycol by *Pseudomonas putida* UV4 [4].

In this paper we determine the general usefulness of ethanol as a carbon source/co-substrate for whole cell biotransformations by comparison of the use of ethanol and glucose for the naphthalene dioxygenase-catalyzed hydroxylation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (NDHD, Figure 1) in a variety of microbial hosts. The use of ethanol as a co-solvent has also been examined, in particular with respect to the effect of increasing ethanol concentration on dissolved substrate and product levels and the maintenance of the volatile substrate within the reactor. In order to make these comparisons across a range of Gram-negative bacteria, the naphthalene dioxygenase gene was cloned into the broad host range vector pMMB66EH [14] forming the plasmid pSS2 [42] and transferred into *Pseudomonas putida* PpG 277, *Pseudomonas aeruginosa* PAC 1R and *Escherichia coli* JM107. Naphthalene dioxygenase is the first enzyme in the naphthalene degradative pathway converting naphthalene to

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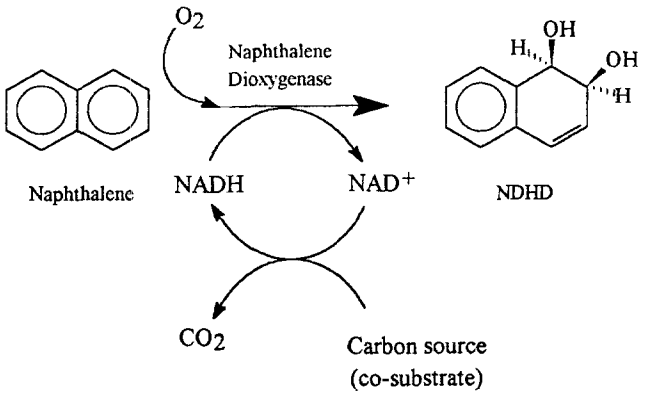

Figure 1 Regiospecific hydroxylation of naphthalene to (+)-*cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene (NDHD) by naphthalene dioxygenase encoded on the pSS2 plasmid.

NDHD [23]. The enzyme requires the presence of molecular oxygen and a carbon source for NADH recycling [9]. Naphthalene dioxygenase is a multi-subunit protein with three redox centres. Present within the cytoplasm, it is sensitive to contact with air and loses activity rapidly outside the cell [5], making use of the enzyme within intact cells essential. Like other microbial dioxygenases, naphthalene dioxygenase has a low substrate specificity [15,37] and will accept a range of aromatic compounds but produces only the *cis* form of the corresponding dihydrodiol [40] making it invaluable as a catalyst for the production of synthetic building blocks [20,29].

Materials and methods

Bacteria and plasmids

The naphthalene dioxygenase *nah* A gene was previously inserted between the *Eco*R1-*Hin*dIII sites of the broad host range vector pMMB66EH [14] forming the plasmid pSS2 [42]. pSS2 was transferred to *Escherichia coli* JM107 (thi$^-$) and *Pseudomonas aeruginosa* PAC 1R using the helper plasmid pRK2013 [11] from *Escherichia coli* HB101 pRK2013 pSS2 [42] and into *Pseudomonas putida* PpG 277 (trp$^-$) from *Escherichia coli* S17-1 pSS2 [43]. Strains were selected by antibiotic resistance to carbenicillin (250 μg ml^{-1}) (Sigma, Poole, Dorset, UK) and nutrient requirements of thiamine (20 μg ml^{-1}) (Sigma) and tryptophan (20 μg ml^{-1}) (Sigma). Active biocatalysts were identified by the formation of indigo from indole (Aldrich, Gillingham, Dorset, UK) after growth on nutrient agar (Oxoid, Basingstoke, Hampshire, UK) containing isopropyl-β-D-thiogalactosyl pyranoside (IPTG) (20 μg ml^{-1}) (Sigma) and subsequent incubation in the presence of indole crystals at 28°C.

Production of active biocatalyst

All cells were grown in 2-L shake flasks containing 500 ml nutrient broth No. 1 (Oxoid) at 250 rpm (28°C). Antibiotics, carbenicillin (Sigma) at 250 μg ml^{-1} and kanamycin (Sigma) at 20–50 μg ml^{-1}, were added as required. Enzyme expression was induced by the addition of IPTG (Sigma) at 20 μg ml^{-1} in early log phase. Cells were harvested at maximum specific activity (*P. putida* PpG 277 – 3 h, *P. aeruginosa* PAC1R – 4 h and *E. coli* JM107 – 3.5 h after induction) by centrifugation at 2744 × *g* for 20 min (28°C). Fresh cells were used for all biotransformations. Prior to use cells were washed once and resuspended in 50 mM, pH 7.0 potassium phosphate buffer.

Biotransformation conditions

All solutions were preincubated at 28°C prior to use. Naphthalene was added to the reactor prior to cell addition at subsaturation concentrations in 50 mM pH 7.0 potassium phosphate buffer (buffer A). Biotransformations were performed with 0.5 g dry weight L^{-1} cells (monitored by optical density readings at 670 nm) resuspended in buffer A containing up to 40 g L^{-1} glucose or ethanol. The total reaction volume was 70 ml. Reactors were maintained at 28°C, aerated at 1–13 vvm and agitated at 750 rpm by a 6-bladed Rushton turbine impeller.

Determination of extracellular naphthalene dihydrodiol concentration

Cell suspensions from biotransformations were centrifuged in a microfuge for 3 min and the supernatant fluid was analysed by HPLC. Naphthalene and naphthalene dihydrodiol concentrations were determined by HPLC using UV detection at 262 nm and separated by a μBondapak C_{18} column (Waters, Milford, MA, USA) and a mobile phase of acetonitrile (450) : water (550) : glacial acetic acid (1) at a flow rate of 1 ml min^{-1} [41]. Retention times for naphthalene and naphthalene dihydrodiol were 17 and 3.4 min, respectively.

Results

Physical effects of ethanol as a co-solvent

Ethanol was added to buffer A at concentrations between 0 and 40 g L^{-1} incubated with an excess of substrate (naphthalene) and product (NDHD) crystals at 28°C. Samples were analysed by HPLC and concentrations determined from the extinction coefficients. NDHD was approximately 2.5-fold more soluble than naphthalene. Ethanol had a minimal effect on the dissolved naphthalene concentration whilst increasing the NDHD concentration in buffer A by approximately 30% at 40 g L^{-1} ethanol (Figure 2).

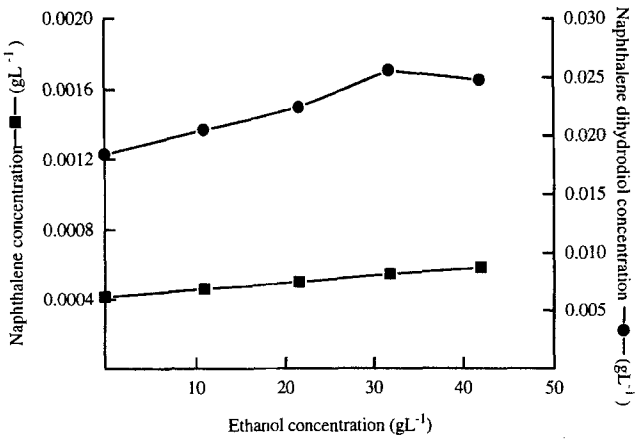

Figure 2 Aqueous saturation concentration of naphthalene (■) and NDHD (●) in the presence of ethanol.

Biological effects of ethanol as a co-substrate

In order to determine the feasibility of using ethanol as both carbon source and co-substrate, biotransformations were undertaken comparing equal mass quantities of ethanol and glucose. Subsaturating concentrations of naphthalene ensured the same substrate supply rates and reduced the possibility of product inhibition. Under aerobic conditions ethanol and glucose would be expected to be fully metabolised to CO_2 and water, regenerating NADH for naphthalene dioxygenase biotransformation. At higher glucose concentrations utilisation may not be as efficient. Under optimum conditions glucose provides (theoretically) about 2.5-fold more NADH than the equivalent molar amount of ethanol. The effect of ethanol compared with glucose on *P. putida* PgG 277 after 30 min exposure is shown in Figure 3. Enzyme activity decreased with increasing amounts of ethanol. After the initial similarity in activities large differences were seen between those metabolising glucose and those using ethanol. Comparisons were made of three Gram-negative bacteria (Figure 4). Both *Pseudomonas* strains showed similar trends with increasing ratio of activities (glucose/ethanol) being seen with increasing ethanol concentration. *E. coli* JM107, however, showed an opposite response with ethanol being more beneficial at high concentrations.

Biological effects of ethanol as a co-solvent

Ethanol as a co-solvent can be toxic to cells; this was highlighted with *P. aeruginosa* PAC 1R. This strain proved to be highly sensitive to ethanol and lysed throughout the biotransformation, losing approximately 90% of cell mass over a 90-min period forming a highly viscous white stringy liquid with large quantities of foam. Neither of the other two strains showed any physical sensitivity to ethanol and no loss of cell integrity over the time period examined.

Biocatalysts – choice

The choice of a biocatalyst for a particular biotransformation is often based on enzyme expression levels alone. However other factors should also be taken into account.

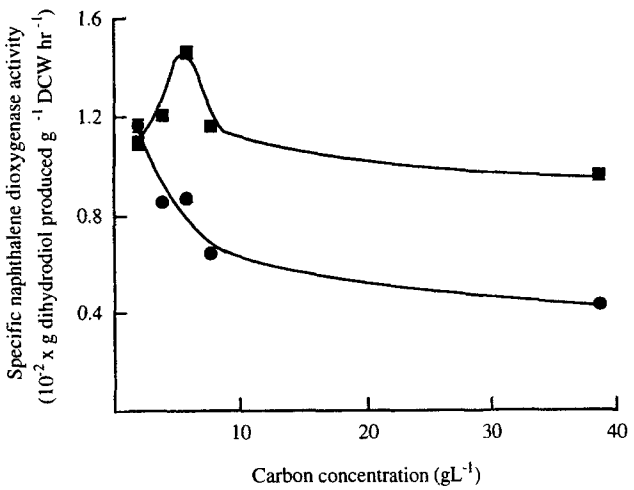

Figure 3 Effect of ethanol (●) and glucose (■) on *P. putida* PpG 277 specific naphthalene dioxygenase productivity (g NDHD g DCW^{-1} h^{-1}) after 30 min.

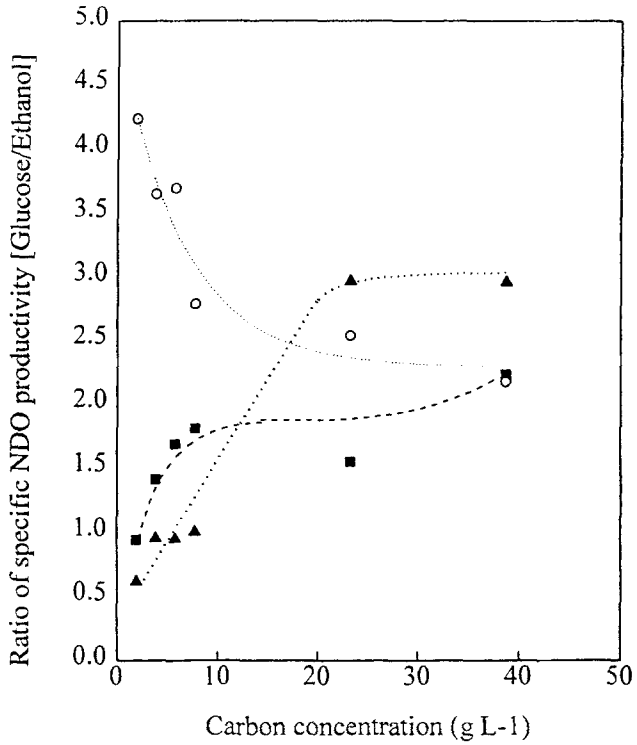

Figure 4 Ratio (glucose/ethanol) of NDO specific productivities (g NDHD g DCW^{-1} h^{-1}) of *P. putida* PpG 277 (■), *P. aeruginosa* PAC 1R (▲) and *E. coli* JM107 (○).

A comparison of specific activities is shown in Table 1. *P. putida* PpG 277 showed the highest activity making it apparently the best catalyst. Enzyme activity will preferably be constitutive or induced by the substrate. Induction using gratuitous inducers such as IPTG are expensive on an industrial scale and can only be justified by large increases in productivity. The level of change in enzyme activity on induction is a reflection of the tightness of molecular control at the promoter. *P. putida* PpG 277 showed an 8.5-fold increase in enzyme activity on induction, *P. aeruginosa* PAC 1R activity increased by 8-fold whilst *E. coli* JM107 only increased 2-fold. NDHD preferentially partitioned away from the cell into the aqueous phase and remained stable in this phase over a period of days at room temperature. Problems arise with some biocatalysts when non-specific side reactions occur. This was found in the case of *P. putida* PpG 277 with the apparent formation of α-naphthol and naphthoquinone when both glucose and ethanol were used as carbon sources.

Table 1 Expression levels of naphthalene dioxygenase in Gram-negative bacteria *P. putida* PpG 277, *P. aeruginosa* PAC 1R and *E. coli* JM107

Strain	Enzyme activity (mg g^{-1} h^{-1})	
	Uninduced	Induced
P. putida PpG 277 pSS2	1.566	13.600
P. aeruginosa PAC 1R pSS2	0.146	1.126
E. coli JM107 pSS2	0.878	1.668

Discussion

Early attempts to understand solvent interaction with microbial catalysts centered on the correlation of the solvents' physical parameters, such as the Hildebrand solubility parameter and dielectric constant, with biocatalyst activity. In order to improve the use of physical parameters, three have been combined and used in the study of an isolated enzyme [39]. Whilst this method was more successful than the single parameter approach, it is far more complicated and time consuming. The most widely used parameter is the solvent Log P value [27]. Related studies using solvents as anaesthetics suggested that efficacy was related to the critical membrane concentration of a solvent (Meyer–Overton Rule) [32] or the critical membrane volume of a solvent [33]. Likewise, the toxicity of solvents to microorganisms has been linked to the solvent membrane concentration and Osborne [34] estimated a theoretical critical membrane concentration of ~0.2 M. The sigmoidal relationship of solvent Log P with retention of biocatalyst activity was thought to reflect this partitioning of a solvent into the membrane and the solubility of a solvent with the aqueous phase, toxicity being apparent with the more hydrophilic solvents log $P \leq 3$. Franks and Lieb [13] attempted to measure the membrane concentration of solvents directly using the firefly luciferase enzyme. However difficulties still ensued in distinguishing between solvent incorporated within and on the surface of membrane fractions. Nevertheless their data indicate that solvents continue to partition into membranes long after the critical concentration was reached implying that the site of action was membrane proteins not lipids as previously suggested. Whilst the molecular interactions leading to biocatalyst inactivation are unclear, several important factors determine a microorganism's solvent tolerance; namely (1) the metabolic state of cells; (2) previous exposure to solvent [19]; (3) the ability to metabolise solvent; and (4) presence of particular genes (Organic Solvent Tolerance – OST) [3] or wide ranging antibiotic resistance [2].

Differences in the solvent tolerance of Gram-positive and Gram-negative bacteria have also been noted [18] with Gram-negative bacteria being more solvent-tolerant. It has been suggested that this is due to the outer membrane of the Gram-negative bacteria. However in studies over a 24-h exposure period no differences were noted between different Gram-negative bacteria on exposure to solvents with a low log P [46]. The work in this study shows two Gram-negative bacteria, namely *P. putida* and *P. aeruginosa*, to be very sensitive to ethanol. Many whole cell bioconversions may not require 24-h batch reaction times (and reuse may be problematic) and therefore it is important to study the initial effect of solvents on these cells.

The use of dissolved amounts of solvents has the potential to increase dissolved substrate and product concentrations which may improve reaction rates hence aiding biocatalysis. Ethanol is of particular interest since it may be oxidatively metabolised using the intracellular alcohol dehydrogenase. This reaction forms the NADH required for naphthalene dioxygenase reaction whilst regenerating NAD maintaining the intracellular NAD/NADH balance enabling further metabolism of acetaldehyde via the TCA cycle [24]. In this particular example the increase in dissolved naphthalene concentration with increasing ethanol concentration is minimal, although the product (NDHD) was seen to increase by approximately 30% at ~4% (w/v) ethanol. The aqueous saturation concentration of NDHD is approximately 2.5-fold that of naphthalene and hence under the operational (substrate at subsaturation levels) conditions used this benefit was not exploited. If naphthalene crystals had been added to the biotransformation medium then higher final aqueous product concentrations would have been reached until product inhibition terminated the reaction, exploiting the use of ethanol. The experiments reported here have determined differences in response to ethanol by the reaction whether through interference of ethanol with the naphthalene dioxygenase directly, through primary metabolic pathways preventing regeneration of cofactors or through disruption of microbial cell walls and cell integrity. Studies on the effect of ethanol on microorganisms to date have focused mostly on the effect of ethanol on yeast with a view to understanding end product inhibition and improving alcohol production [21]. However the effects of ethanol on prokaryotic and eukaryotic microorganisms are very similar [24] and involve interference in cell wall synthesis, membrane leakage, growth, macromolecular synthesis and often involve alteration of cell wall/membrane composition including fatty acid chain length [19], the level of saturation and *cis/trans* ratio [22] and hence membrane fluidity [1]. It has also been proposed that ethanol interacts with lipid membranes at the phospholipid hydrophilic head group in reverse micelles dehydrating the membrane [6].

Biocatalysts are selected initially on the basis of expression of required enzyme activity, preferably with low substrate specificity but high regioselectivity and sterospecificity. Problems arise if the natural strain is unstable or where substrate degradation or side chain reactions occur. Degradation of product not only leads to reduced total productivity but also to the presence of structurally related compounds making purification difficult, increasing processing time and cost. In these cases recombinant DNA (rDNA) techniques may be used to isolate and transfer the required genes to better defined host strains [25]. The choice of microbial host for use as a (rDNA) biocatalyst is based not only on enzyme expression and activity levels but also on the levels of resistance to substrate and product inhibition and the ability to produce biocatalyst reproducibly [26]. rDNA techniques may be used not only to improve enzyme expression but also to alter protein structure with a view to understanding enzyme action and improving substrate range or stereospecificity. Unfortunately these techniques are time consuming and the use of rDNA strains has the added regulations and health risks associated with them. Use of biocatalysts with solvents requires a good understanding of the interaction of the solvent with the catalyst particularly with strains prone to lysis and emulsion formation. Ideally the required enzymes will be expressed constitutively without the requirement for antibiotic selection hence reducing production costs. The selection of one particular host is complex and a compromise between each of the above factors.

It is of interest that as carbon concentrations increase,

the relative activities (glucose/ethanol) of *P. putida* PpG 277 and *P. aeruginosa* PAC 1R increase whilst *E. coli* JM107 decreases indicating that at high concentrations ethanol may be of particular use with *E. coli*.

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