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REVIEW

Dioxygenase- and monooxygenase-catalysed synthesis of *cis*-dihydrodiols, catechols, epoxides and other oxygenated products

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Abstract Oxidoreductases are an emerging class of biotechnologically relevant enzymes due to their regio- and stereo-specificity. The selective oxygenation of aromatic compounds by oxidoreductases has received much attention and a wide range of reactions have been documented using these enzymes from various microbial sources. This review gives an overview of various dioxygenase, monooxygenase and oxidase enzymes that have been manipulated for the synthesis of products such as *cis*-dihydrodiols, catechols, epoxides and other oxygenated products. The use of protein engineering and its advancement in the synthesis of recombinant enzymes is also discussed.

Keywords Biocatalysis · Dioxygenase · Directed evolution · Monooxygenase · Oxidase

Introduction

Numerous reviews have documented the microbial metabolism of natural and synthetic hydrocarbons (Lavallee et al. 2005; Wentzel et al. 2007; Husain 2008). Many of these studies have highlighted that aerobic metabolism generally involves an extended series of enzymatic conversions of hydrocarbons to aliphatic intermediates of major metabolic pathways. Arising from these investigations are a wide range of microorganisms and enzymes many of which are now being investigated for the production of value added compounds (Parales et al. 2002; Ahuja et al. 2004). Biocatalysis has become an important technology for the production of fine chemicals and pharmaceutical synthons (van Beilen and Li 2002; van Beilen et al. 2003). While enzyme-catalysed reactions are not without their problems, it is widely recognised that traditional organic synthesis of fine chemicals can often be lengthy with energy intensive multi-stepped reactions (Azerad 2001; Tao et al. 2004a, b). Some reactions require expensive starting materials and/or equipment due to the elevated temperatures and pressures at which they operate. In addition the formation of undesirable side products in chemical synthesis requires a clean up process resulting in low product yields and high costs (Azerad 2001; Tao et al. 2004a, b).

Enzymes are not limited to catalysing reactions with their natural substrate and often show activity towards a range of structurally related compounds (Faber 2004). Typically the rates of enzyme-mediated processes are accelerated by a factor of 10^7–10^{12} compared to those of the corresponding non-enzymatic reactions (Menger 1993; Faber 2004; Hibbert and

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Dalby 2005). In addition, enzymes carry out a wide range of regio-, stereo- and chemo-specific reactions under mild conditions (Azerad 2001; Li et al. 2002; van Beilen and Li 2002; Burton 2003; van Beilen et al. 2003; Faber 2004). These properties alone have been exploited for the production of optically pure compounds and for the development of efficient routes to target compounds that may not be synthesised otherwise by chemical means (Duetz et al. 2001; Huisman and Gray 2002; van Beilen et al. 2003). The advantages of biocatalysis for the production of pure compounds has been recognised by organic chemists and the production of valuable chemicals such as acrylamide, polyacetic acid and chiral synthons using biocatalysis has been developed to industrial scale (Schmid et al. 2001a; Straathof et al. 2002; van Beilen and Li 2002).

Oxidoreductase enzymes are the largest and most diverse group of enzymes and a large number of them use molecular oxygen as a substrate (oxygenase) or as an electron acceptor (oxidase) (Bugg 1997; Wackett and Hershberger 2001). These enzymes have activity towards a wide range of substrates and display high regio- and stereo-specificity (Li et al. 2002; van Beilen and Li 2002; Burton 2003; van Beilen et al. 2003).

Oxygenases are ubiquitous in nature and play an important role in the metabolism of a broad range of compounds (Wackett and Hershberger 2001; Burton 2003; Ullrich and Hofrichter 2007). Monooxygenases catalyse the incorporation of one atom of O_2 into the organic substrate and the other atom is reduced to water. These enzymes generally use NADH or NADPH cofactors to provide reducing potential for the supply of electrons to the substrate and can be metal-, haem- or flavin-dependent (Bugg 1997; Burton 2003; Ullrich and Hofrichter 2007). In dioxygenase reactions both atoms from a single molecule of O_2 are incorporated into the organic substrates (Burton 2003; Boyd and Bugg 2006; Boyd et al. 2006b; Ullrich and Hofrichter 2007). Dioxygenases include two major classes: haem-dependent iron sulphur dioxygenases and Rieske iron-sulphur non-haem dioxygenases the majority of which are NADH dependent (Wackett and Hershberger 2001; Burton 2003).

Oxidase enzymes catalyse the oxidation of a substrate without incorporating oxygen directly into the product (Burton 2003; Ullrich and Hofrichter 2007). These enzymes react with molecular oxygen in a two-electron oxidation process to produce hydrogen peroxide that subsequently acts on the target substrate. These enzymes are often metal-, haem- or flavin-dependent and many of the oxidases are capable of catalysis without the exogenous addition of cofactors (Burton 2003; Seo et al. 2003; Claus and Decker 2006; Ullrich and Hofrichter 2007).

One of the most challenging and fundamental reactions in organic synthesis is the regiospecific hydroxylation of the aromatic ring (Canada et al. 2002; Burton 2003; Vardar and Wood 2004; Di Gennaro et al. 2005; Fishman et al. 2005; Tao et al. 2005a; Ullrich and Hofrichter 2007). The ability of oxygenases and oxidases to regiospecifically hydroxylate the aromatic ring or sidechain substituent of an aromatic substrate presents opportunities for biocatalysis to synthesize compounds that are difficult to synthesize using organic synthesis (Duetz et al. 2001; Li et al. 2002; Burton 2003; Ullrich and Hofrichter 2007). Over the past few years numerous applications and opportunities for a wide range of these enzymes have been described in books and numerous papers (Azerad 2001; Duetz et al. 2001; Wackett and Hershberger 2001; Schmid et al. 2001a; Huisman and Gray 2002; Li et al. 2002; van Beilen and Li 2002; Burton 2003; van Beilen et al. 2003; Ahuja et al. 2004; Faber 2004; Patel 2007; Urlacher and Schmid 2007). This review however focuses on the use of particular bacterial dioxygenases, monooxygenases and oxidases for the synthesis of substituted *cis*-dihydrodiols, catechols, epoxides and other hydroxylated compounds with potential as fine chemicals and pharmaceutical synthons. Enzymes such as the alcohol dehydrogenases, amino acid dehydrogenases, Baeyer-Villiger and FAD-dependent non-heme monooxygenases, to name just a few others, are not discussed in this article (Faber 2004; Patel 2007; Ullrich and Hofrichter 2007; Urlacher and Schmid 2007).

The biological synthesis of *cis*-dihydrodiols

The initial step in the biodegradation of numerous aromatic compounds by aerobic bacteria often involves the incorporation of O_2 into the aromatic ring (Di Gennaro et al. 2005; Boyd and Bugg 2006; Boyd et al. 2006b; Boyd et al. 2007). This dihydroxylation reaction is catalysed by dioxygenase enzymes resulting in the formation of chiral *cis*-dihydrodiol products.

While *cis*-dihydrodiol intermediates are generally stable, dehydrogenase enzymes present in bacteria catalyse the dehydrogenation of *cis*-dihydrodiols to catechol derivatives (Boyd and Bugg 2006; Boyd et al. 2007). Mutant strains containing the dioxygenase enzyme, but lacking the dehydrogenase enzyme activity, as well as recombinant *Escherichia coli* strains expressing the dioxygenase enzyme have been developed with a view to synthesising a range of *cis*-dihydrodiol products (Boyd and Bugg 2006; Lee 2006; Boyd et al. 2006b).

Toluene dioxygenase (TDO) of *Pseudomonas putida* UV4 and 39/D strains is one of the most widely studied dioxygenase enzyme with a view to biocatalyst development (Boyd and Bugg 2006; Boyd et al. 2007). In addition to toluene and benzene, this enzyme has activity towards a broad range of mono-substituted arenes with high regio- and stereo-specificity (Boyd and Bugg 2006; Boyd et al. 2007). Using recombinant strains expressing TDO, numerous *cis*-dihydrodiol products have been synthesised from mono-cyclic arenes (Table 1). In fact greater than three hundred 2,3-*cis*-dihydrodiol derivatives have been synthesised from substituted benzene substrates, the majority of which are enantiomerically pure (Boyd et al. 2006b). The most widely produced *cis*-dihydrodiol synthons are the 2,3-*cis*-dihydrodiol derivatives of chlorobenzene, bromobenzene and toluene (Boyd and Bugg 2006). These synthons are used in the synthesis of natural target molecules including (−)-cladospolide A, (−)-*ent*-bengamide E and 6C-methyl-D-mannoses (Table 1) (Boyd and Bugg 2006).

In addition to mono-substituted arenes, TDO has activity towards a range of compounds such as alkyl-substituted benzenes (Fig. 1a), acyclic and cyclic conjugated alkenes (Fig. 1b), disubstituted benzenes (Fig. 1c), substituted styrenes (Fig. 1d), substituted phenyl methyl sulfides (Fig. 1e) and alkyl-substituted pyridine substrates (Fig. 1f) (Boyd et al. 2006b). Oxidation of these compounds occurs on the arene ring forming a range of interesting diols. However depending on the stereodirecting effects of the alkyl sidechain substituent oxidation can occur on the sidechain resulting in the formation of alcohols (Fig. 1g) and sulfoxides (Fig. 1h) (Boyd et al. 2006b). These alcohols are often substrates for TDO and are further oxidised to triols (Fig. 1i) or they may also undergo spontaneous decomposition to the corresponding aldehyde (Fig. 1j) (Boyd et al. 2006b). Studies with di-substituted compounds have shown that TDO oxidises *ortho*- and *para*-substituted alkylbenzenes exclusively to their corresponding enantiopure 2,3-*cis*-dihydrodiols (Fig. 1k, l). However, oxidation of the *meta* di-substituted equivalents results in the formation of benzylic alcohol products in addition to the *cis*-dihydrodiol derivative (Fig. 1m) (Boyd et al. 2006b). Understanding the stereo-directing and *meta* effects of substituents is of paramount importance to realising biocatalytic potential as it allows for the development of a model that can be used to predict key products of future TDO oxidation reactions (Boyd et al. 2006b).

As a general rule, TDO can only accommodate mono-cyclic substituted substrates and poly-cyclic arenes no larger that naphthalene due to the size of the enzyme active site (Boyd and Bugg 2006; Boyd et al. 2006a). The more polar mono-substituted arenes such as benzoic acid, aniline and benzene sulphonic acid are not reported as substrates for TDO and the synthesis of 1,2- or 3,4-*cis*-dihydrodiols has not been observed for TDO (Boyd and Bugg 2006; Boyd et al. 2006b). Despite the successes of TDO its limitations as a biocatalyst have given rise to studies with other dioxygenase enzymes.

Biphenyl-dioxygenase (BDO) and naphthalene-dioxygenase (NDO) can accommodate larger carbocyclic and heterocyclic arenes (Boyd and Bugg 2006). Both BDO and NDO catalyse the dihydroxylation of substrates including naphthalene, anthracene and phenanthrene to *cis*-dihydrodiol products (Boyd and Bugg 2006; Lee 2006; Boyd et al. 2006a). Indeed naphthalene has been used in the production of (+)-goniodiol from naphthalene *cis*-1,2-dihydrodiol (Table 1) (Boyd and Bugg 2006). NDO at present is unique in its ability to oxidise biphenyl (Fig. 1n) to its corresponding 3,4-*cis*-dihydrodiol (Fig. 1o) (Boyd and Bugg 2006). While this compound accumulates as a minor product site-directed mutagenesis has resulted in a NDO mutant that favours the formation of the 3,4-*cis*-dihydrodiol as the major product. Benzoic acid dioxygenase (BZDO) and nitrobenzene dioxygenase (NBDO) have been reported to synthesise 1,2-*cis*-dihydrodiols (Fig. 1r, s) from benzoic acid (Fig. 1p) and nitrobenzene (Fig. 1q) respectively (Boyd and Bugg 2006). The varied oxidising activities of these enzymes together with the steric and *meta* directing effects of substituents allows for the synthesis of a wide range of 1,2-, 2,3- and 3,4-*cis*-dihydrodiols that is not possible by any other means.

Table 1 Physiologically significant microbial enzymes for the preparation of fine chemicals

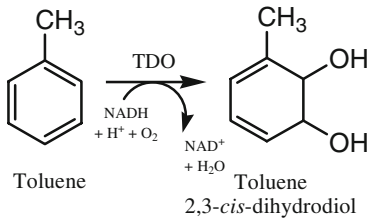
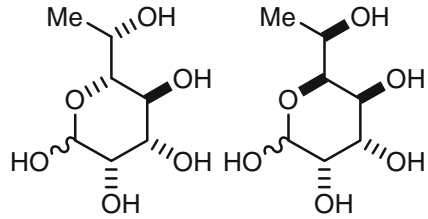
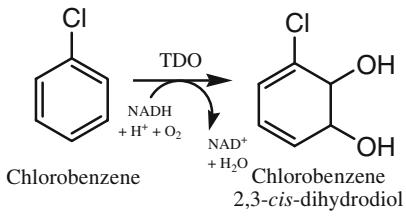
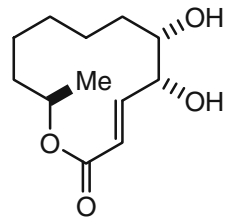
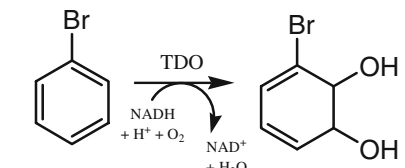
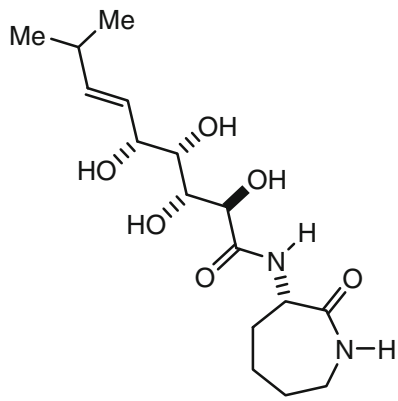
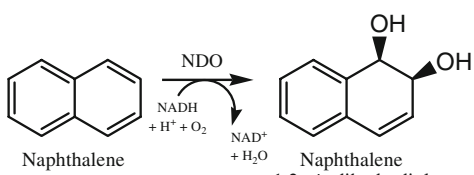
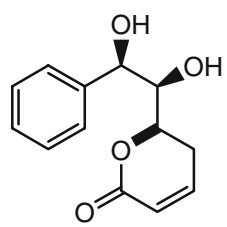
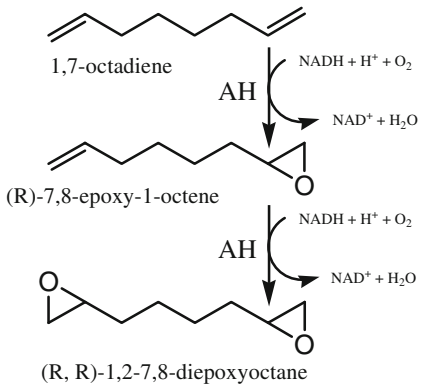
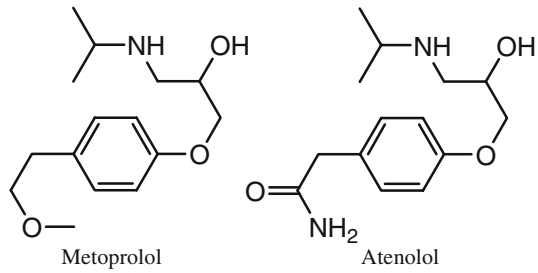
Microorganism/ Enzyme activity	Relevant industrial *cis*-dihydrodiol synthons	Formation of end product from *cis*-dihydrodiol
Pseudomonas putida UV4 Toluene dioxygenase (TDO)	 Toluene Toluene 2,3-*cis*-dihydrodiol	 7 steps to 6C-Methyl-D-mannose
	 Chlorobenzene Chlorobenzene 2,3-*cis*-dihydrodiol	 11 steps to (-)-Cladospolide A
	 Bromobenzene Bromobenzene 2,3-*cis*-dihydrodiol	 11 steps to (-)-*ent*-Bengamide E
Pseudomonas putida 9816/11 Naphthalene dioxygenase (NDO)	 Naphthalene Naphthalene 1,2-*cis*-dihydrodiol	 11 steps to (+)-Goniodiol

Table 1 continued

Microorganism/ Enzyme activity	Relevant industrial *cis*-dihydrodiol synthons	Formation of end product from *cis*-dihydrodiol
Pseudomonas oleovorans Alkane hydroxylase (AH)		

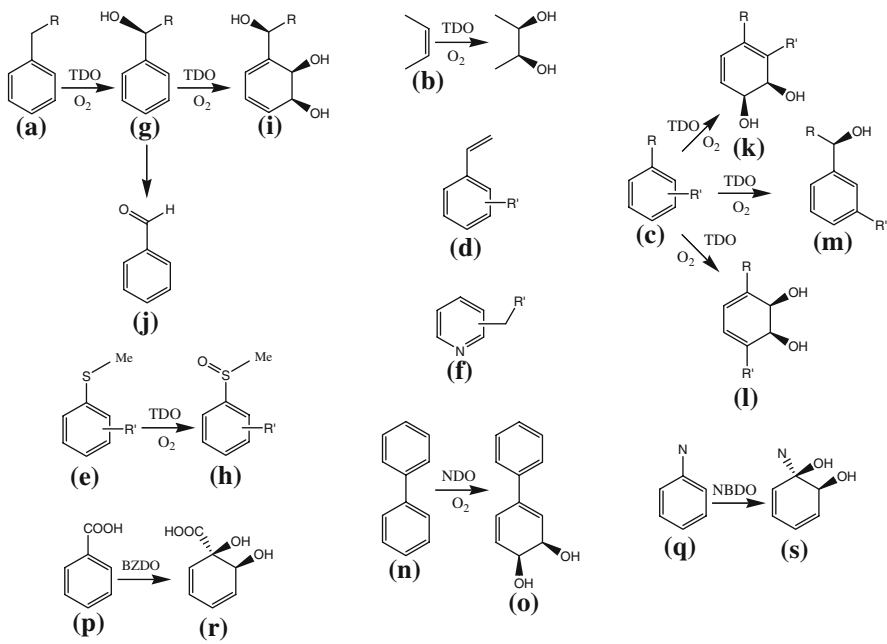


Fig. 1 The enzymatic synthesis of a range of 1,2-, 2,3- and 3,4-*cis*-dihydrodiols using dioxygenase enzymes

The biosynthesis of substituted catechols

2,3-Substituted catechols

In addition to dihydrodiols, dioxygenases are key biocatalysts for the synthesis of other important synthons. Substituted catechols are important aromatic compounds and are widely used as synthons in industrial processes such as the manufacture of plastics, polymers, drugs and dyes (Allouche et al. 2004; Tao et al. 2004a, b). In combination with dehydrogenases, dioxygenases have been explored in terms of their ability to synthesise a range of substituted 2,3-catechols (Bui et al. 2000; Berberian et al. 2007). Using a recombinant organism expressing the first two enzymes in the biodegradation of toluene by *Pseudomonas* species, namely TDO and dihydrocatechol dehydrogenase (DHCD), several 2,3-substituted catechols have been synthesised from substituted arenes (Table 2) (Bui et al. 2000). These

Table 2 Microbial enzymes for the preparation of synthons with potential industrial applications

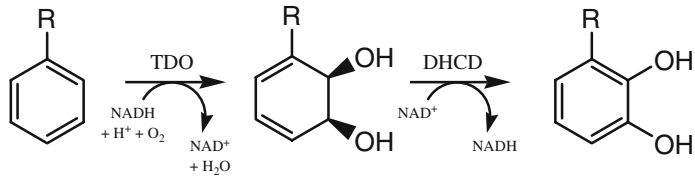
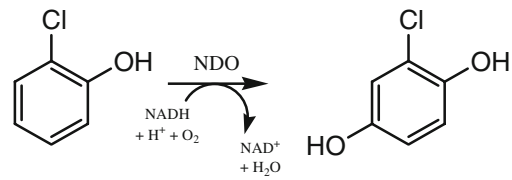
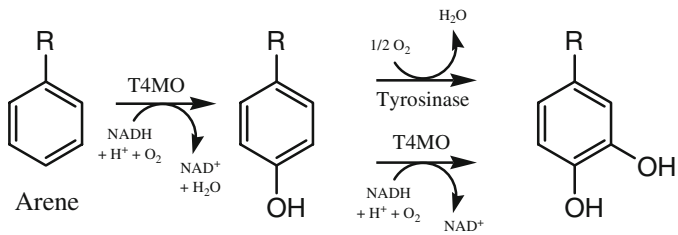
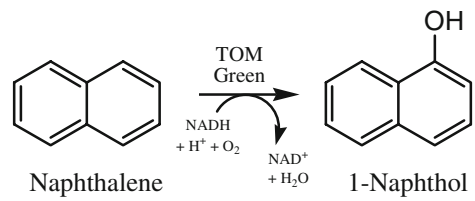
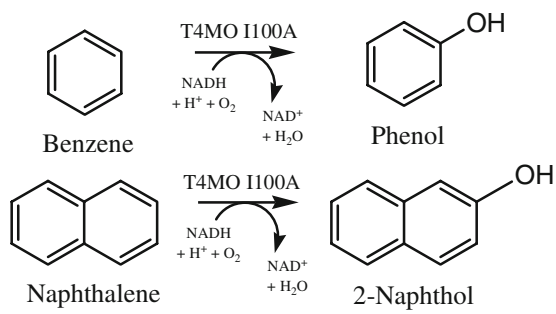
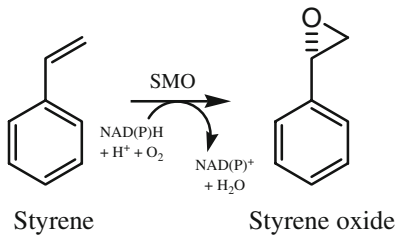
Microorganism/Enzyme activity	Potential industrial synthons
Pseudomonas species Toluene dioxygenase (TDO) Dihydrocatechol dehydrogenase (DHCD)	 2,3-Substituted catechols
Pseudomonas NCIB 9816-4 Naphthalene dioxygenase (NDO)	 2-Chlorophenol Chlorohydroquinone
Mushroom Tyrosinase *Pseudomonas putida* F6 Bacterial Tyrosinase *Pseudomonas mendocina* KR1 Toluene-4-monooxygenase (T4MO)	 Arene 4-Substituted Phenol 3,4-Substituted Catechol
Escherichia coli TG1 TOM-Green	 Naphthalene 1-Naphthol
Escherichia coli TG1 Toluene-4-monooxygenase (T4MO) T4MO I100A variant	 Benzene Phenol Naphthalene 2-Naphthol

Table 2 continued

Microorganism/Enzyme activity	Potential industrial synthons
Pseudomonas putida S12 *Mycobacterium* strain E2 *Pseudomonas* sp. strain VLB120 Styrene monooxygenase (SMO)	

catechols include 2,3-chloro-, 2,3-bromo- and 2,3-iodo-catechol and are useful synthons for organic chemists (Bui et al. 2000). NDO also catalyses the mono-hydroxylation of substituted phenols to yield dihydroxybenzenes including 3- and 4-substituted catechols and substituted hydroquinones (Lee 2006). In addition it exclusively oxidises 2-chlorophenol to chlorohydroquinone (Table 2). This product is an important precursor used in pharmaceutical and other organic synthetic processes (Lee 2006).

3,4-Substituted catechols

3,4-Substituted catechols, such as L-DOPA (3,4-dihydroxyphenylalanine), adrenaline and noradrenaline, are well known for their biological activities. Phenol oxidases, particularly tyrosinase, have been explored in terms of their biocatalytic potential to synthesise substituted 3,4-catechols (Burton 2003; Seo et al. 2003; Claus and Decker 2006; Halaouli et al. 2006). Tyrosinase (E.C. 1.14.18.1) is a copper-containing enzyme and is ubiquitous in nature. Much of the information on the structure and function of tyrosinase has been obtained from studies on tyrosinase from mushroom species such as *Agaricus biosporus* (Seo et al. 2003; Halaouli et al. 2006). In addition, information on tyrosinase from *Streptomyces antibioticus*, *Neurospora crassa* and a number of fruits and vegetables has also been obtained (Espín et al. 2001; Burton 2003; Seo et al. 2003; Claus and Decker 2006; Halaouli et al. 2006). Tyrsoinase uses O_2 to catalyse two reactions: (1) the *ortho*-hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and (2) the oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity) which are subsequently polymerised to red/brown melanin like pigments (Burton 2003; Seo et al. 2003; Claus and Decker 2006; Halaouli et al. 2006). The biocatalytic potential of tyrosinase has received much attention as this enzyme has the advantage of being readily available, has a broad substrate range, can be used in organic media and does not require the addition of exogenous cofactors (Pialis and Saville 1998; Espín et al. 2001; Burton 2003; Seo et al. 2003; Brooks et al. 2004).

Studies on the use of mushroom tyrosinase for the synthesis of L-DOPA, the drug of choice for the treatment of Parkinson's disease, from L-tyrosine has been reported (Pialis and Saville 1998). The production of L-DOPA was enhanced using mushroom tyrosinase immobilised on chemically modified nylon-6,6 membranes and resulted in the formation of 33 ± 0.6 mg l^{-1} h^{-1} in a 500 ml system. This production rate remained constant during scale up to 1 l and 2 l bioreactors by keeping the enzyme concentration constant (Pialis and Saville 1998).

In addition to L-DOPA, hydroxytyrosol (Hty) [2-(3,4-dihydroxyphenyl)-ethanol] has also been synthesised from tyrosol [2-(4-hydroxyphenyl)-ethanol] using mushroom tyrosinase under reducing conditions (Espín et al. 2001). Hty is described as a powerful antioxidant and inhibits low density lipoprotein oxidation, scavenges free radicals and has antimicrobial activity towards Gram positive and Gram negative bacteria (Allouche et al. 2004). This compound has also been synthesised using tyrosinase present in cell extracts of *Pseudomonas putida* F6 immobilised in a calcium alginate matrix (Brooks et al. 2005). The immobilisation of the enzyme resulted in a 21% increase in product formation

compared to the non-immobilised enzyme. Furthermore immobilisation of the enzyme increased stability and longevity and allowed for reuse of the biocatalyst (Brooks et al. 2005). Resting cells of *Pseudomonas aeruginosa* pregrown on tyrosol produced Hty (96% yield) from 4 g tyrosol l^{-1} over 7 h (Allouche et al. 2004). While the enzyme responsible for the catalytic conversion remained unidentified the whole cell system has several advantages over the enzymatic system using mushroom or bacterial tyrosinase. These include the low cost of producing the enzyme, the increased stability of the enzyme within the cell, no requirement for a reducing agent to prevent quinone formation and, with a 96% product yield, low clean up costs (Allouche et al. 2004).

Although the formation of catechols using biocatalysis is interesting phenolic compounds are expensive and thus cheaper starting materials maybe required for industrial applications. The conversion of substituted benzenes to catechols via phenols could reduce the cost of biotransformation processes. A number of toluene degrading bacteria expressing monooxygenase enzymes are capable of converting toluene and other substituted arenes to *o*-, *m*- and *p*-substituted phenols (Rui et al. 2004; Vardar and Wood 2004; Fishman et al. 2004b; Tao et al. 2004b). These phenol products can be converted to their catechols derivatives through successive hydroxylation by the same enzymes (Rui et al. 2004; Vardar and Wood 2004; Fishman et al. 2004b; Tao et al. 2004b). Initial studies reported that T4MO catalysed the first hydroxylation of toluene to predominantly *p*-cresol and further metabolism of this intermediate was catalysed by subsequent enzymes in the pathway (sidechain) attack (Whited and Gibson 1991). However, during investigations with recombinant strains expressing T4MO, it was shown that T4MO can catalyse the sequential hydroxylation of benzene, nitrobenzene and fluorobenzene to catechol, 4-nitrocatechol and 4-fluorocatechol, respectively, via their respective phenolic derivatives (Fishman et al. 2004a; Tao et al. 2004b; Nolan and O'Connor 2007). These findings are significant as nitrocatechols are useful precursors for the synthesis of pharmaceutical drugs, such as Flesinoxan, while fluorinated compounds have the ability to influence biological activity (e.g. enzyme inhibition, affect energy generation processes and cell-to-cell communication) (Fishman et al. 2004a; Natarajan et al. 2005).

Although the formation of 4-nitrocatechol from nitrobenzene accounts for less than 5% of the total product formed (Fishman et al. 2004a, 4-fluorocatechol is the sole product formed from fluorobenzene depending on the biotransformation conditions used (Nolan and O'Connor 2007). In an attempt to improve 4-fluorocatechol formation, the combination of T4MO and tyrosinase for the conversion of fluorobenzene to 4-fluorocatechol was demonstrated by our research group (Table 2) (Nolan and O'Connor 2007). T4MO catalysed the first hydroxylation step (fluorobenzene to 4-fluorophenol) and tyrosinase catalysed the second hydroxylation step (4-fluorophenol to 4-fluorocatechol). While this tandem biotransformation is interesting, the oxidation of the catechol product to *o*-quinones by the diphenolase activity of tyrosinase is undesirable. This oxidation step can be prevented by the addition of reducing agents such as ascorbic acid. However, such measures increase the overall cost of the process (Nolan and O'Connor 2007).

Biosynthesis of epoxides

Enantiopure epoxides are valuable intermediates in the synthesis of a number of optically active pharmaceutical compounds (McClay et al. 2000; Schmid et al. 2001b; Mooney et al. 2006). Epoxides have been used for the production of β3-adrenergic receptor agonists, anti-obesity drugs, nematocides and anticancer agents (Lee and Shuler 2007). The asymmetric epoxidation of olefinic compounds and the enantioselective resolution of racemic epoxides using chemocatalysts are a few of the approaches to the chemical synthesis of these important synthons (Lee and Shuler 2007). Styrene monooxygenase (SMO) catalyses the formation of styrene epoxide from styrene (Table 2) (Panke et al. 2000; Schmid et al. 2001b; O'Leary et al. 2002; Otto et al. 2004; Mooney et al. 2006). This reaction has been used as a model reaction for the development of styrene monooxygenase as a broad range biocatalyst for the synthesis of epoxides. Studies with SMO have shown that both the (*S*)- and (*R*)-enantiomers of styrene oxide can be produced depending on the microbial source of the enzyme (Nöthe and Hartmans 1994; Wubbolts et al. 1994; Panke et al. 1999; Panke et al. 2000; Park et al. 2006). Nöthe and Hartmans (1994) showed that the *P. putida* S12 mutant M2 produced

predominantly the (*S*)-isomer while cells of *Mycobacterium* strain E2 produced the (*R*)-isomer. In each case one enantiomer predominated with enantiomeric excess values of 93 to 98%. (*S*)-Styrene oxide is a useful building block in the synthesis of nematocide levamisole thus a two liquid-phase fed-batch process was developed for the production of this isomer using *E. coli* JM101 cells expressing SMO genes (*styAB*) of *Pseudomonas* sp. strain VLB120 (Panke et al. 2000, 2002; Park et al. 2006). Using bis(2-ethylhexyl) phthalate as the organic phase under the 2 litre fed-batch conditions, 11 g of (*S*)-styrene oxide (>99% ee) was produced in 10 h. In addition dioctyl/phthalate has been used as an organic solvent for the synthesis of a range of substituted epoxides from substituted styrenes (Bernasconi et al. 2000; Schmid et al. 2001b). The use of an organic phase has many advantages including: (1) in situ product recovery preventing product toxicity/enzyme inhibition; (2) acts as a reservoir for toxic substrates; and (3) aids in product retrieval after the biotransformation process (Bernasconi et al. 2000; Panke et al. 2000; Schmid et al. 2001b; Park et al. 2006).

Other oxygenase enzymes have been reported as producing epoxides from a range of compounds. These include xylene oxygenase (XO) from *P. putida* mt-2 (Wubbolts et al. 1994), *p*-cymene monooxygenase (CMO) from *P. putida* F1 (Nishio et al. 2001), T4MO (McClay et al. 2000) and alkane hydroxylase (AH) from *P. oleovorans* (May et al. 1976; Archelas and Furstoss 1997). XO produces (*S*)-styrene oxide from styrene to an ee of 93 ± 3% (Wubbolts et al. 1994). While CMO also produces styrene oxide form styrene, this enzyme oxidises 4-chlorostyrene to 4-chlorostyrene oxide at a faster rate (Nishio et al. 2001). However the use of this 4-chlorostyrene oxide as a synthon remains unknown. T4MO expressed in *E. coli* oxidises a range of short-chain alkanes producing epoxide derivatives (McClay et al. 2000). The enantiopurity of the products formed varied dramatically from 54 to 90% of a single isomer. AH converts 1,7-octadiene to (R)-7,8-epoxy-1-octene with an enantiomeric purity of 92% (Table 1). This product, in turn, is further oxidised by AH to (*R*,*R*)-1,2-7,8-diepoxyoctane with an enatiomeric purity of 83% (Table 1) (May et al. 1976; McClay et al. 2000). These observations led to industrial applications for the synthesis of the drugs Metoprolol and Atenolol (Table 1) (Archelas and Furstoss 1997; McClay et al. 2000).

Limitations to enzyme regiospecificity

T4MO activity expressed in whole cells or as a cell free system exhibits a high degree of regiospecificity with toluene, nitrobenzene and methoxybenzene as substrates (Fishman et al. 2004a; Tao et al. 2004a, b). The predominant regioisomer (90–100%) is the *para*-substituted phenol with minor amounts of *meta*- and *ortho*-regioisomers formed. Research carried out by our group using *P. mendocina* KR1 and *E. coli* whole cells heterologously expressing wild type T4MO demonstrated the formation of 2-(3-hydroxyphenyl)-ethanol [2(3HP)E] and 2-(4-hydroxyphenyl)-ethanol [2(4HP)E] from 2-phenylethanol (2PE) as a substrate with the 3-substituted phenol predominating (Fig. 2a). The ethanol substituent is *meta*-directing and, while

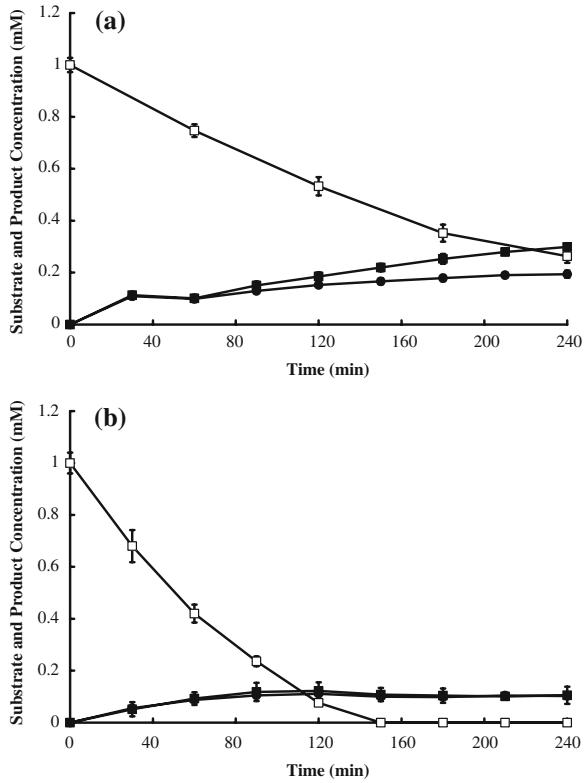


Fig. 2 Substrate depletion and product formation in the biotransformation of (**a**) 2PE (1 mM) by whole cells of *Pseudomonas mendocina* KR1 (1.5 mg CDW/ml) expressing T4MO activity (□, 2PE; ● 2(4HP)E; ■ 2(3HP)E) and (**b**) substrate depletion and product formation in the biotransformation of EB (1 mM) by whole cells of *Pseudomonas mendocina* KR1 (0.3 mg CDW/ml) expressing T4MO activity (□, EB; ■, 4EP; ●, 3EP)

at first sight, this may explain our observation that the nitro moiety of nitrobenzene is also *meta*-directing but *para*-nitrophenol is the predominant regioisomer formed (Fishman et al. 2004a). To determine the effect of a similar sized moiety that is *ortho*- and *para*-directing rather than *meta*-directing, ethylbenzene was used as a substrate for whole cells expressing T4MO activity.

Under similar biotransformation conditions using EB as substrate approximately equimolar amounts of 3-ethylphenol (3EP) and 4-ethylphenol (4EP) were formed in the reaction medium (Fig. 2b). Thus biocatalysts (enzymes) such as T4MO can suffer from poor regiospecificity when challenged with substrates other than their natural substrate. While the results above suggest the use of T4MO to synthesise 4-substituted phenols is limited, protein engineering to enhance the regiospecificity of T4MO could potentially generate an array of enzyme variants that are tailor made to synthesise a single regioisomer from a specific substrate. Furthermore it opens up the opportunity to engineer enzymes to synthesise 3-substituted phenols.

Protein engineering

The engineering of protein composition generally occurs through site directed or random (error prone PCR) mutagenesis and is often employed to modify a target enzyme with a view to altering activity, specificity and stability (Joo et al. 1999; Canada et al. 2002; Hibbert et al. 2005; Hibbert and Dalby 2005). The random mutagenesis approach is a powerful tool for the development of enzymes as biocatalysts as enzyme variants with improved activity can be generated in a relatively short period of time. The key advantages of engineered biocatalysts are the ability to synthesis compounds at increased rates and specificities thus saving on energy and production costs (Joo et al. 1999; Canada et al. 2002; Hibbert et al. 2005; Hibbert and Dalby 2005).

Protein engineering for 1- and 2-naphthol synthesis

Naphthols are widely used in the manufacturing of dyes, herbicides and insecticides. 1-Naphthol is presently produced at 15,000 tons per year in the United States (Canada et al. 2002). The demand for 2-naphthol (50,000 tons per year) is 3 times greater than that of 1-naphthol (Tao et al. 2005a). The chemical synthesis of 2-naphthol is made difficult due to the high concentrations of dangerous acids used such as naphthalene-1-sulfonic acid, multi-stepped reactions, extreme temperatures (−68°C to −78°C) to obtain high selectively and the use of metal catalysts (Tao et al. 2005a). No known domestic producer of 2-naphthol exists in the United States since 1982 and it is thought that production of 2-naphthol will decrease in China as environmental protection policies increase. This, in turn, will lead to the global shortage of this valuable compound and thus highlights the importance of developing biocatalysis for chemical synthesis (Tao et al. 2005a).

Random mutagenesis was used to evolve the multicomponent TOM enzyme for multiple biocatalytic functions (Canada et al. 2002). One of these functions was to improve oxidation of naphthalene for the production of naphthol. The TOM shuffled library was screened using whole cells in a 96-well plate with one mutant that had significantly increased synthetic rates and yields of 1-naphthol selected for further study. This mutant, named TOM-Green due to its inherited ability to turn green in LB medium, formed 7.6-fold more naphthol from naphthalene compared to the wild type enzyme (Table 2) (Canada et al. 2002). In addition the product ratio of 1-naphthol to 2-naphthol was 97–3%, respectively, thus indicating that DNA shuffling did not alter the regiospecificity of the mutant enzyme. DNA sequence analysis confirmed that DNA shuffling resulted in the introduction of four base pair changes to the gene sequence encoding TOM. One of these base pair changes led to one amino acid change (V106A) in the α-subunit of the hydroxylase component (TomA3) (Canada et al. 2002).

Based on structural modelling of TOM the majority of mutations that improve the specificity of an enzyme generally occurs within 10 Å of the enzyme active site with the smaller side-chain of alanine in the TOM-Green V106A variant allowing greater access of substrate to the catalytic site compared to valine (Canada et al. 2002; Hibbert and Dalby 2005). Due to the relatedness of TOM to T4MO and based on the improved rate of naphthalene oxidation by the TOM-Green variant saturation mutagenesis was focused at the analogous position

TmoA I100 of T4MO as well as random mutagenesis (Canada et al. 2002; Tao et al. 2005a, b). Wild type T4MO oxidises naphthalene producing 50% 1- and 2-naphthol (Tao et al. 2005b). T4MO variants, I100L and G103S/A107G, hydroxylated naphthalene to favour the formation of 1-naphthol to purity levels of 87% and 99%, respectively, while variants I100A, I100S and I100G favoured the formation of 2-naphthol to purity levels of 88–99% from naphthalene (Tao et al. 2005b). The creation of such diversity demonstrates the power of protein engineering to generate variants of a single protein with the ability to produce different regioisomers of the same compound.

Tao et al. (2005a) developed a two-phase system for the biosynthesis of (1) phenol from benzene, and (2) 2-naphthol from naphthalene using whole cells expressing the T4MO I100A variant. Phenol, in addition to naphthol, is also used widely (6.6×10^6 tons per year) as a precursor in the manufacturing of dyes, drugs, perfumes, insecticides and surfactants (Tao et al. 2005a). A two-phase system has several advantages over the single-phase aqueous system. These include increasing substrate solubility and reducing substrate/product toxicity. It also prevents the undesired successive hydroxylation reaction that results in the formation of catechol from benzene and dihydroxynaphthalene from naphthalene in the single phase system. Under these conditions 10- and 21-fold higher concentrations of phenol (1.88 g/l at 97% purity) and 2-naphthol (2.88 g/l at 92% purity) were obtained, respectively, compared to the single phase system (Table 2) (Tao et al. 2005a). The yield of product formed in these biotransformations compares well with the commercial synthetic counterpart. The scale-up of this process like many others is the challenge for biocatalysis.

Other active site residues, including I100, Q141, T201 and F205, have been identified as contributing to the regiospecific activity of the T4MO enzyme (Tao et al. 2004a). Analogous residues in TouA α-hydroxylase fragment of ToMO have become the focus of site directed mutagenesis for a number of groups with a view to developing a biocatalyst for the synthesis of nitrocatechol, nitroquinone, methylhydroquinone, methylresorcinol and pyrogallol all of which are commercially important (Vardar and Wood 2004; Fishman et al. 2004a; Tao et al. 2004a).

Conclusions

Oxidoreductases are becoming increasingly important for the synthesis of new and important compounds. Indeed biocatalysis has replaced several high-volume chemical processes due to the regio-, stereo- and chemo-specific activity of enzymes (van Beilen et al. 2003; Vardar and Wood 2004; Fishman et al. 2004a). Despite the successes and knowledge of hundreds of oxidoreductase enzymes only few make it to industrial scale. The development of recombinant enzymes using directed evolution techniques is a stepping stone in over coming some of the problems such as low rates of activity and, in some cases, poor regio- and stereospecificity (Hibbert et al. 2005; Hibbert and Dalby 2005). As the majority of oxidoreductases are cofactor-dependent, multi-component enzymes the development of host strains for the stable expression of these enzymes is critical. Furthermore the ability of the host cells to tolerate organic solvents is important for process development and scale-up as substrate solubility and product inhibition in an aqueous environment are key issues that have arisen in recent reports (Bernasconi et al. 2000; Panke et al. 2000; Schmid et al. 2001b; Tao et al. 2005a; Park et al. 2006). Oxidoreductases will find application in the short term in niche markets when the advantages offered by these enzymes outweigh the advantages of conventional synthetic routes. In the end, market economics will dictate whether a biocatalyst succeeds or not.

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