



UNIVERSITY OF INSUBRIA

Ph.D. School in Biological and Medical Sciences

Ph.D. Program in
Analysis Protection and Management of Biodiversity
- XXIV Course -

BACTERIAL OXYGENASES: FEATURES, POTENTIAL AND NEW INVESTIGATION METHODS

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Ph.D. thesis of

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2008-2011

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Abstract

Bacterial oxygenases, the enzymes involved in the breakdown pathways of aromatic and aliphatic hydrocarbons, have aroused great interest within the scientific community for their potential applications in a number of different fields: environmental (bioremediation and biomonitoring), biotechnological (green chemistry) and biomedical (drug production).

The aims of this study were 1) to clone new bacterial oxygenases using conventional methods and further develop new screening techniques and 2) to assess the ability of the newly-cloned enzyme(s), of toluene/*o*-xylene monooxygenase (*To*MO) and of some of its mutants, to exploit various types of aromatic substrate on which the activity of these enzymes has never previously been assessed.

Using conventional techniques, *Pseudomonas* sp. N1 genes coding for naphthalene dioxygenase (NDO) and *Sphingobium* sp. PhS genes, putatively coding for Phenanthrene dioxygenase (PhDO), were cloned; of the two enzymes only NDO appeared to be functional. *To*MO is an enzyme complex formed from six different subunits, ABCDEF; subunit A (*Tou*A) is part of the terminal hydroxylase and contains the active site. In this study, in addition to wild type enzymes, the *Tou*A mutants D211A and D211A/E214G, previously created by this laboratory, and *Tou*A E214G, already described in the literature, were also used.

The ability of *To*MO and *Tou*A mutants, and of NDO to hydroxylate different aromatic compounds other than their natural substrates (toluene and naphthalene respectively) was evaluated by biotransformation assays using, as substrates, the following molecules: 1,2,3-trimethoxybenzene, anisole, benzophenone, bibenzyl, biphenyl, nitrobenzene, quinoline, and *trans*-stilbene. These compounds were chosen for the environmental, biotechnological and pharmacological importance of their hydroxylated derivatives. To this end, *To*MO, *Tou*A mutants and NDO were expressed in the heterologous host *E. coli* JM109. For both enzymes, the products of bioconversion were identified by GC-MS and, using the appropriate standard of reference, quantified by HPLC analysis.

*To*MO proved active in all substrates tested. The mutant E214G, like the wild type, was capable of oxidising all the compounds tested: in almost all the biotransformations both the mutant form and the wild type behaved in the same way in that they both produced the same isomers in similar proportions. In terms of catalytic efficiency, the mutant E214G was found to be most powerful: the mutants D211A and D211A/E214G were capable of

hydroxylating only a few of the substrates used (1,2,3-trimethoxybenzene, anisole, benzophenone, biphenyl) with very low yields. However, considering the fact that strains expressing the mutants D211A e D211A/E214G showed impaired growth and expression problems, probably attributable to unwanted mutations introduced during mutagenesis, these data cannot be considered definitive.

The ability of NDO to perform dihydroxylation processes was confirmed but, in addition, monohydroxylation reactions (quinoline) and dealkylation reactions (anisole and 1,2,3-trimethoxybenzene) were observed. The quantitative analysis of the biotransformation products showed that the enzyme efficiency decreased with the increasing steric footprint of the substrate. The failure of nitrobenzene to be transformed suggests that, in addition to steric footprint, other factors such as the presence of deactivating substituent groups plays a key role in the catalytic process of this enzyme.

Both NDO and T_oMO have shown a good potential in the field of biotransformation and further studies could lead to the production of more useful and efficient enzyme variants.

During an internship at the University of Granada in Spain, a new protocol, based on the SIGEX (Substrate Induced Gene Expression) system, was developed to screen bacterial genomic libraries. This method, which is normally used to screen metagenomic libraries, was adapted to analyse libraries created from a single bacterial genome. With this technique it is possible to perform a rapid screening on the basis of a signal emitted from a reporter gene included in the construct used in creating the library. Using a previously constructed library, this approach allowed, in a short time, 60 clones to be isolated that had a high probability of containing naphthalene-dependent regulatory genes. The sequencing of the obtained clones, a necessary step in order to validate the protocol, is currently in progress.

1. Introduction

1.1. The metabolic potential of microorganisms

The disposal of aromatic and aliphatic organic compounds, whether naturally occurring or man made, is a problem of global concern with important political and economical implications.

The chemical industry, with the production of polymers, combustibles, solvents, pesticides and pharmaceuticals, has released and continues to release into the environment vast amounts of 'xenobiotics' (from the Greek *xenos*; stranger), that is to say, synthetic compounds with a different structure to that of a naturally occurring molecule. In the broadest sense, the term can also be used for those compounds that, although of a natural origin, are not normally present in any appreciable quantities in the biosphere such as, for example, petroleum. Ecological disasters, where the accidental loss of compounds resistant to biodegradation (e.g. the oil spill from the tanker Haven in the Bay of Genova in 1991; the sinking of the tanker Prestige off the coast of Galicia in 2004), of course, do not help the above situation. In this scenario of environmental emergencies, associated with development that has now become unsustainable, the world of microorganisms plays a crucial role [1].

Microorganisms, with their vast natural repertoire of metabolic and genetic diversity, the result of a long and complex evolutionary process, constitute a huge reservoir of biodiversity that has been, and still has, a major role in the maintaining balance within the biosphere [2]. Over the course of evolution, bacteria have developed a great ability to adapt to different environmental conditions, managing to exploit many different chemical compounds as a source of carbon and energy [3]. Nevertheless, while molecules that have always been present in the environment, such as monocyclic aromatic hydrocarbons, are degraded by numerous microorganisms, xenobiotic compounds, as well as many polycyclic aromatic hydrocarbons (PAHs), are often resistant to biodegradation: the former because they only came into contact with microorganisms around one hundred years ago, too short a time period to allow the evolution of metabolic pathways that are capable of degrading them, the latter because of their structural complexity.

In relatively recent times, particular bacterial strains have been isolated that are capable of using complex aliphatic and aromatic hydrocarbons as a source of carbon and energy, converting them into intermediates of their central metabolism [4, 5]. The microorganisms already described, of which the numbers are continually increasing, have aroused great interest in the scientific community for their potential application in different areas of

biotechnology. These include bio-remediation of contaminated sites, biotransformation of toxic compounds into less harmful products or products of high industrial value (*green chemistry*), and the development of bio-sensors for the monitoring of pollutants. The range of substrates on which these microorganisms are able to grow is extensive and includes both toxic compounds and those that are difficult to degrade, such as benzene, toluene, ethylbenzene, xylenes (BTEX), a number of substituted phenols, polycyclic aromatic hydrocarbons (PAHs), and chlorinated organic compounds [4, 6, 7].

In recent years, the presence of a large number of contaminated sites has led to the rapid development of soil remediation technologies. Among them, the one that has aroused most interest is biological degradation. The choice of technique to be used for the remediation of a contaminated site is determined by many factors according to the nature of the pollutants, the site in question, the technology itself, and the economic and legal evaluations. In the case of contamination by hydrocarbons or organic substances, *ex-situ* biological treatments represent an effective and low-cost intervention. Where microbial communities capable of carrying out the breakdown process are already present within the site, it is possible to enrich, *in-situ*, the species responsible for this process, mainly through the addition of limiting inorganic nutrients and oxygen (biostimulation) [3]. In areas lacking naturally-occurring microorganisms capable of metabolising xenobiotic compounds, the introduction of isolated biodegrading strains from other sites (bioaugmentation) is necessary. Bioaugmentation is conditional on the ability of the selected strains to survive in indigenous microbial communities; often the microorganisms used in this process are derived from selective enrichment cultures and, therefore, are not representative of the indigenous microflora of the recipient site. For this reason, after being inoculated on site, they survive for only a brief period of time as they are unable to compete effectively for nutrients [8].

Bacterial strains specialised in the metabolism of such compounds can be produced in the laboratory, both naturally by selecting, in the presence of contaminants, mutants or recombinant strains, and by using genetic engineering techniques that allow the desired characteristics to be transferred into the appropriate host and/or the implementation of targeted modifications. The use of recombinant bacterial strains is, however, limited to confined areas (bioreactors) because current European legislation does not allow the introduction of genetically modified microorganisms into the natural environment [9]. Bioremediation procedures, compared to traditional chemical decontamination methods, are environmentally friendly, exhibit a low energy consumption and are low in cost [10]

Furthermore, the study of these molecules' catabolic processes has shown that microorganisms and enzymes involved in the breakdown stage, are also tools that can be used effectively in a different biotechnological area, namely bioconversion. The partial degradation of a large number of organic compounds often leads to the formation of intermediates with high added value [11]. In addition, the enzymes involved in one or more stages of the bacterial degradation pathways usually also show activity on compounds that are structurally similar to natural substrates and are, therefore, available for the formation of derivative compounds that are of great industrial and biotechnological interest [12, 13]. Therefore the ability of bacteria to adapt themselves to be able to exploit new chemical compounds, such as growth substrates, is a subject of great and growing interest, not only for the potential applications in the field of bioremediation but also in bioconversion [14]. Industrial processes for the synthesis of fine chemicals, that involve purified microorganisms or enzymes, are steadily increasing [15]. It is estimated that, by the year 2050, 30% of the production processes of the entire global chemical industry will be based on biocatalysis [16]. The environmental impact of biocatalytic processes is extremely small, as they require milder reaction conditions compared to traditional chemical processes, they reduce the need for, potentially dangerous, strong acids and bases, they generally require low concentrations of metals and, usually they don't require the use of solvents. They also involve the formation of fewer undesirable products, reducing the energetic demand and increasing the safety of the process [11].

Finally, it should be remembered that microorganisms and enzymes involved in the breakdown stages of metabolism can also lead to the development of biosensors for the detection of common environmental pollutants. One example is represented by the genetically modified strain *P. fluorescens* HK44 [17, 18]. This strain is able to detect the presence of an environmental contaminant by emitting a bioluminescent signal, allowing it to be used as an effective *in-situ* monitoring tool [18, 19].

It is evident that the use of specific enzymes in biotechnological applications, both for bioremediation and for industrial biocatalysis requires, as a prerequisite, an extensive knowledge of the genetic and biochemical characteristics of the microbial catabolic pathways involved.

1.2. Microbial metabolic pathways

The breakdown of aromatic compounds can occur both aerobically and anaerobically, through energetic metabolic processes, or through co-metabolic processes in which the microorganisms receive neither energy nor a source of carbon. This latter process is particularly important in the breakdown of mixtures of aromatic and PAH compounds of a high molecular weight [20]. Although these compounds have long been considered recalcitrant to breakdown under anoxic conditions, it has been observed that, in fact, breakdown does happen under these conditions, albeit in a slow and inefficient way. Certain members of the genus *Clostridium* are, in fact, able to promote the dehalogenation of polychlorinated biphenyls in anaerobic conditions [21]; another example is the bacterial strain EbS7 isolated from marine sediment samples and belonging to the subclass δ of the proteobacteria, which can, anaerobically, completely mineralise ethylbenzene by linking its oxidation to the reduction of sulphates [22].

In aerobic conditions, however, the breakdown of aromatic compounds is much quicker and more efficient due to the ability to activate stable molecules using molecular oxygen. The aerobic catabolic pathways can be divided into the following functional segments [23]: the processing of substituent groups; transformation of aromatic substrates into dehydroxylated intermediates; the opening of the ring and production of linear compounds containing 2 or 4 carbon atoms; entry of the products into the tricarboxylic acid (TCA) cycle .

Although microorganisms possess a wide variety of enzymes for the initial attack on a number of different compounds, the catabolic pathways usually converge: the substrates are converted into a small number of key intermediates that are subsequently metabolised by a single central pathway. This results in simpler control units and a more efficient metabolic process.

The initial stage of oxidation of an organic compound is catalysed by an oxygenase that, in the presence of molecular oxygen, is able to attack the aromatic ring of the molecule, leading to the formation of dihydroxylated compounds such as catechols, gentisate and protocatechuate (*upper pathway*). These dihydroxylated intermediates can be cleaved by ring-opening oxygenases to give linear compounds (*lower pathway*) that can then enter the TCA cycle. The aromatic ring can be opened by the *meta cleavage pathway*, characterised by the extradiol opening of the ring, or by the *ortho cleavage pathway*, in which the ring is opened intradiol, that is between the two hydroxyl groups (Fig. 1.1) [25].

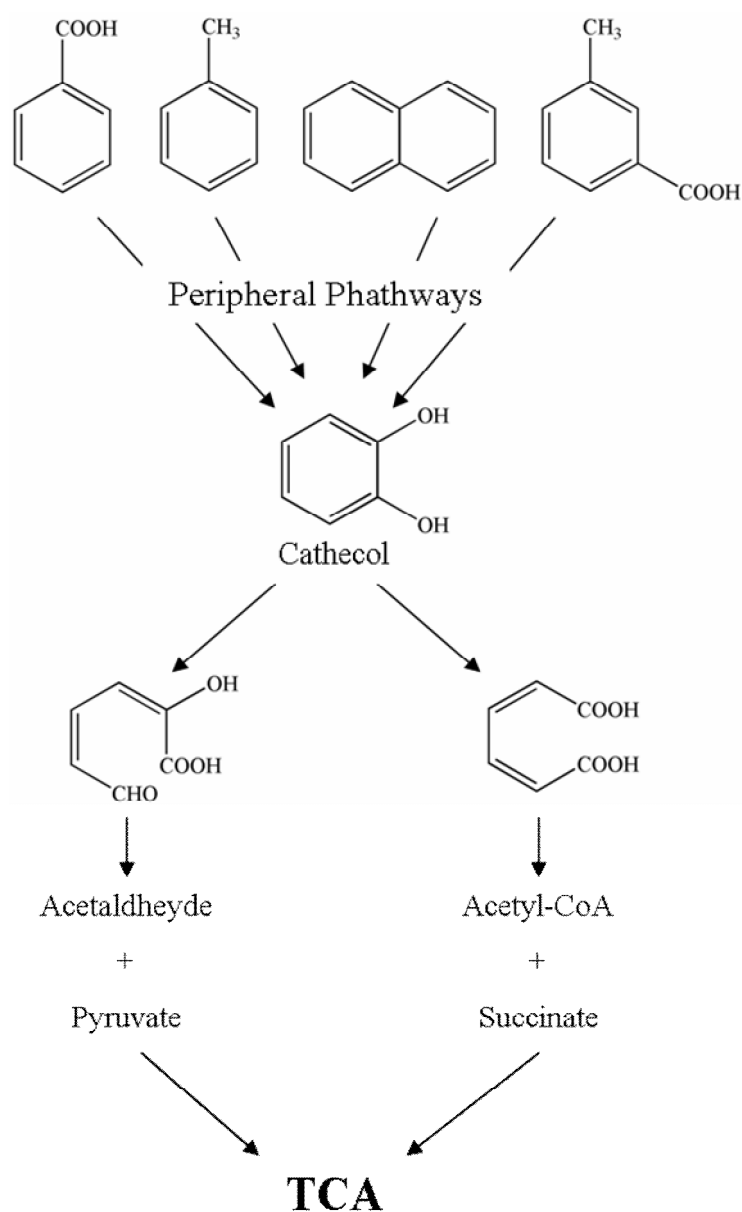


Fig. 1.1. General outline of the breakdown of aromatic compounds.

In the last few decades, more than 100 species have been isolated, belonging to different genera, that are able to breakdown these aromatic compounds [4]. The largest number and the most specialised species belong to the genus *Pseudomonas* [26, 27]; to a lesser extent the genera *Ralstonia*, *Mycobacterium*, *Polaromonas*, *Streptomyces*, *Sphingomonas*, amongst others, also possess similar breakdown capabilities [28, 29].

Bacteria belonging to the genus *Pseudomonas* represent the most remarkable group of microorganisms capable of using, in aerobic conditions, many aromatic and aliphatic compounds as the sole source of carbon and energy. These are the ubiquitous gram-negative bacteria, present in soil and water and capable of establishing symbiotic relationships with higher organisms. These bacteria are capable of breaking down a wide variety of highly aromatic pollutants including BTEX, PAHs, and substituted phenols, as well as extremely cytotoxic compounds such as polychlorinated biphenyls (PCBs). This great metabolic versatility, attributable to the low substrate specificity of the catabolic enzymes, allows these microorganisms to break down structurally related molecules using the same pathways. Frequently, the genes coding for enzymes involved in the catabolism of aromatic compounds are found on mobile genetic elements, such as transposons and plasmids, which facilitate horizontal genetic transfer and the rapid adaptation of the microorganisms to new pollutants. Gene transfer, sometimes interspecific, mediated by processes such as conjugation and transduction, increases the genetic variability of microorganisms and favours the evolution of new catabolic pathways [30]. Recombination events lead to the creation of mosaics of catabolic operons, which can provide the microorganisms with completely new metabolic properties suitable for metabolising new molecules [31, 32, 33].

1.3. Bacterial oxygenases

Oxygenase enzymes play a key role in the breakdown of aliphatic and aromatic hydrocarbons. These enzymes are able to catalyse the introduction of oxygen into organic compounds from simple oxygen molecules (O_2) [23]. The types of oxygenase are of great importance to the industrial sector as they can be used in the chemical synthesis, “*green chemistry*”, of useful products in pharmaceutical and agrochemical fields [34]. The oxygenases can be divided, on the basis of the reaction mechanism, into monooxygenase and dioxygenase.

Monooxygenase catalyses the introduction of a hydroxyl group into the substrate, which can be an aromatic or aliphatic compound. In this reaction, the reduction of two oxygen atoms is seen: one reduced to a hydroxyl group, the other to a water molecule, all in conjunction with the oxidation of NAD(P)H. Monooxygenase is primarily involved in the “upper pathway”.

In aerobic environments, dioxygenase catalyses both the initial NAD(P)H-dependent hydroxylation reaction of the aromatic ring (upper pathway), facilitating the simultaneous introduction of the two hydroxyl groups into the ring, and the reactions that lead to the opening of the aromatic ring (lower pathway) without the intervention of external reducing agents [35] (Fig. 1.2).

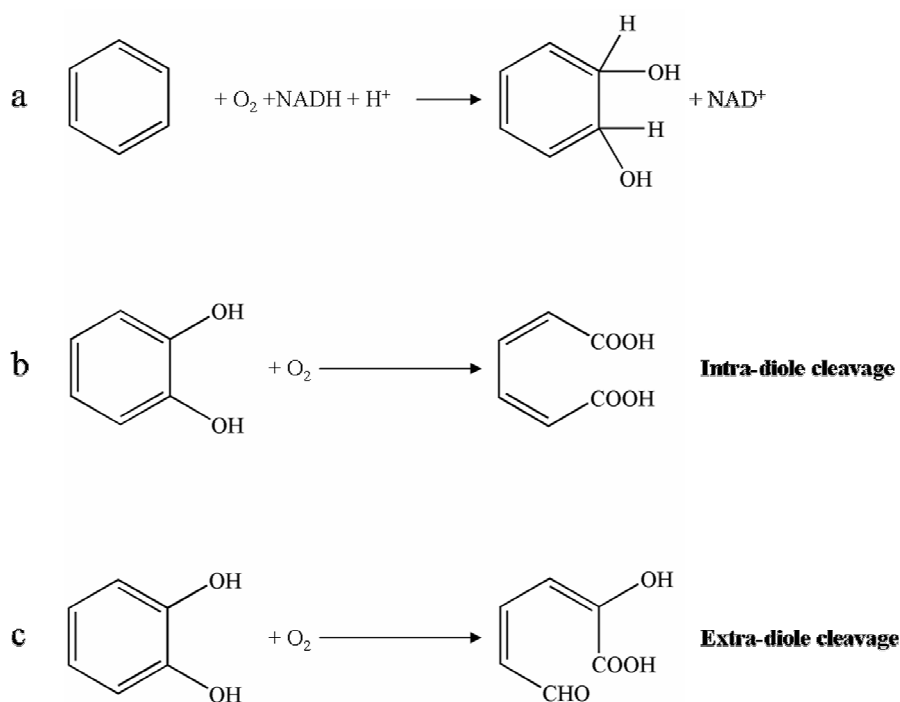


Fig. 1.2. a) Dioxygenase activation reactions of an aromatic ring incorporating two hydroxyl groups at the expense of O_2 and $NADH$. b) and c) reactions involving dioxygenase opening the aromatic ring.

Enzymes belonging to the family of bacterial oxygenases are, often structurally similar, multi-component systems that show, however, notable differences in their substrate range and in the regio-specificity of oxygenation of the aromatic ring [36]. These systems are composed of multiple functional components organised into a short electron transfer chain from NAD(P)H to oxygen. Usually component I is an NAD(P)H-dependent flavoprotein oxyreductase, component II is a ferredoxin containing an iron sulphur centre, while component III is a terminal hydroxylase that contains the active site (usually a monoferric or diferric centre) (Fig. 1.3). The transport of electrons starts with the transfer of one (for monooxygenase) or two (for dioxygenase) electrons from NAD(P)H to the flavin, followed by the transfer of single electrons to the Fe-S centres then to iron on the terminal hydroxylase component, which is usually composed of large oligomeric proteins [35]. In

some monooxygenase enzymes, additional subunits have been identified that are, apparently, indispensable for catalysis although their role is not always entirely clear.

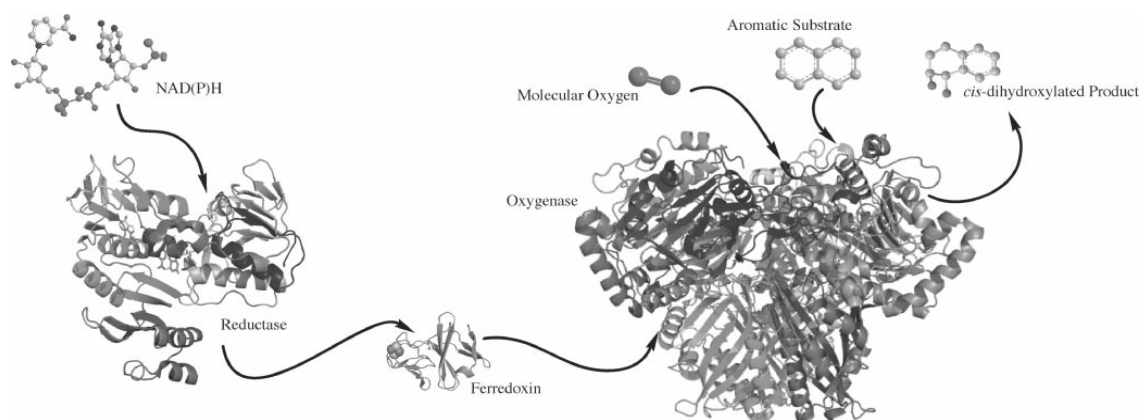


Fig. 1.3. Schematic representation of the components of a bacterial oxygenase.

1.4. Metabolism of naphthalene

Naphthalene is a bicyclic aromatic hydrocarbon that is commonly found in the environment and is often used as a model molecule for studying the breakdown pathways of PAHs as it is the simplest of its class. Many bacteria belonging to the genera *Alcaligenes*, *Burkholderia*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, and *Streptomyces* have been isolated that use naphthalene as the sole source of carbon and energy [29]. One of the best characterised enzyme systems responsible for the breakdown of naphthalene is encoded by the plasmid NAH7 in *Pseudomonas putida* G7: NAH7 has two operons that contain the genes responsible for the breakdown of naphthalene [27].

In the first stage of naphthalene breakdown (Fig. 1.4), two groups of OH are introduced in positions 1 and 2 of the aromatic ring to form cis-(1R,2S)-dihydroxy-1,2-dihydroxynaphthalene (Fig. 1.5) (cis-dihydrodiol of naphthalene) by a naphthalene dioxygenase (NDO). The subsequent stages proceed as shown in Figure 1.5, until catechol or gentisic acid is produced, which is further oxidised to obtain linear compounds that are able to enter the TCA cycle.

In several cases, it has been observed that enzymes involved in the oxidation of naphthalene are able to break down phenanthrene and anthracene to 1-hydroxy-2-naphthoate and 2-hydroxy-3-naphthoate, respectively (Fig. 1.6). The catabolic pathways of other PAHs are essentially similar although, due to the high number of aromatic rings present, the number of steps required to obtain the linear compounds is increased.

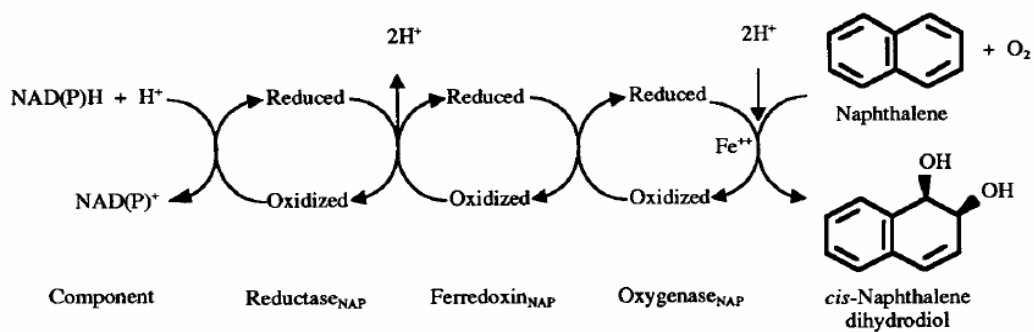


Fig. 1.4. Initial oxidation of naphthalene to cis-1,2-dihydroxy-1,2-dihydroxyaphthalene by naphthalene dioxygenase.

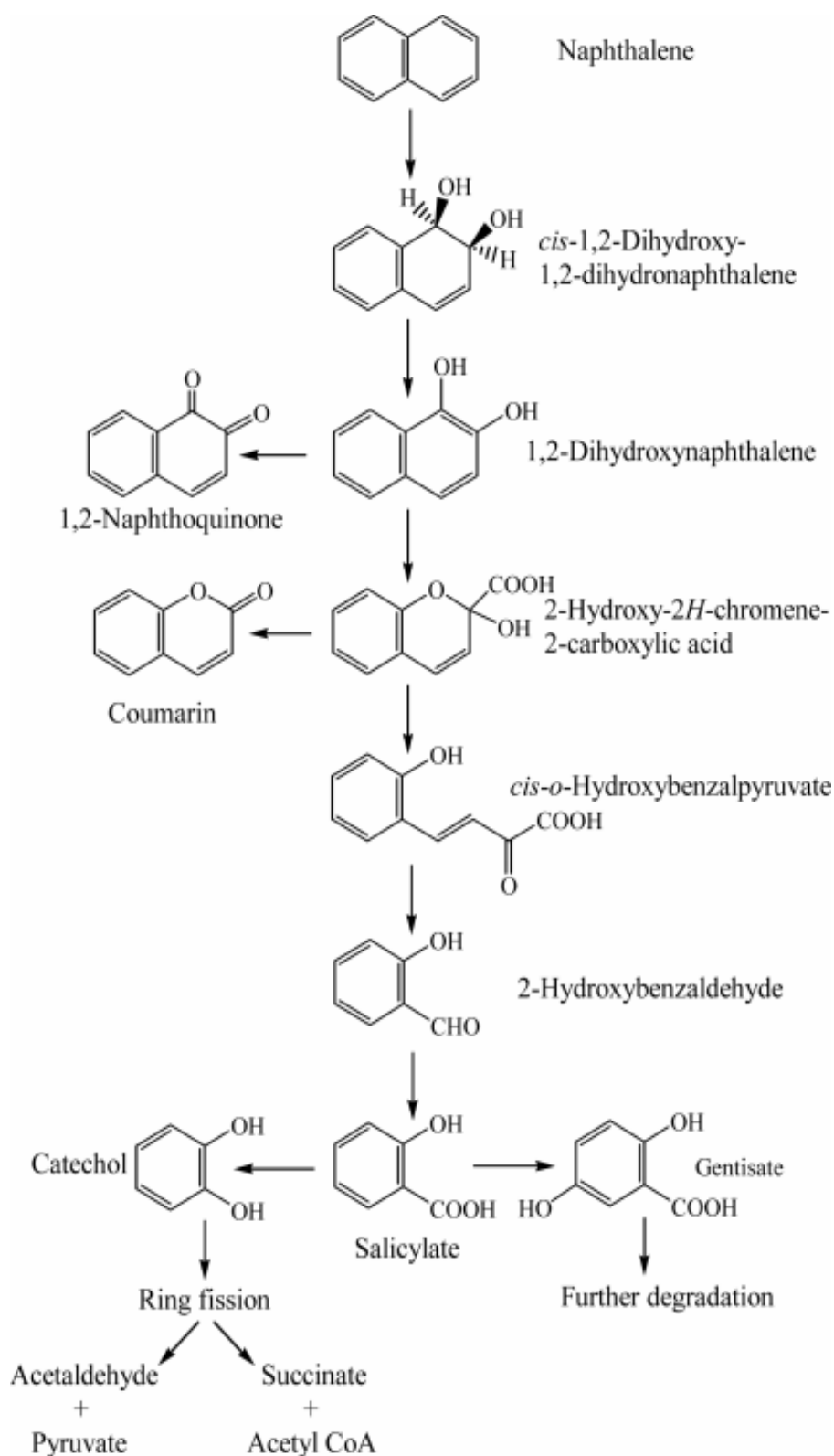


Fig. 1.5. General schematic of the breakdown pathway of naphthalene.

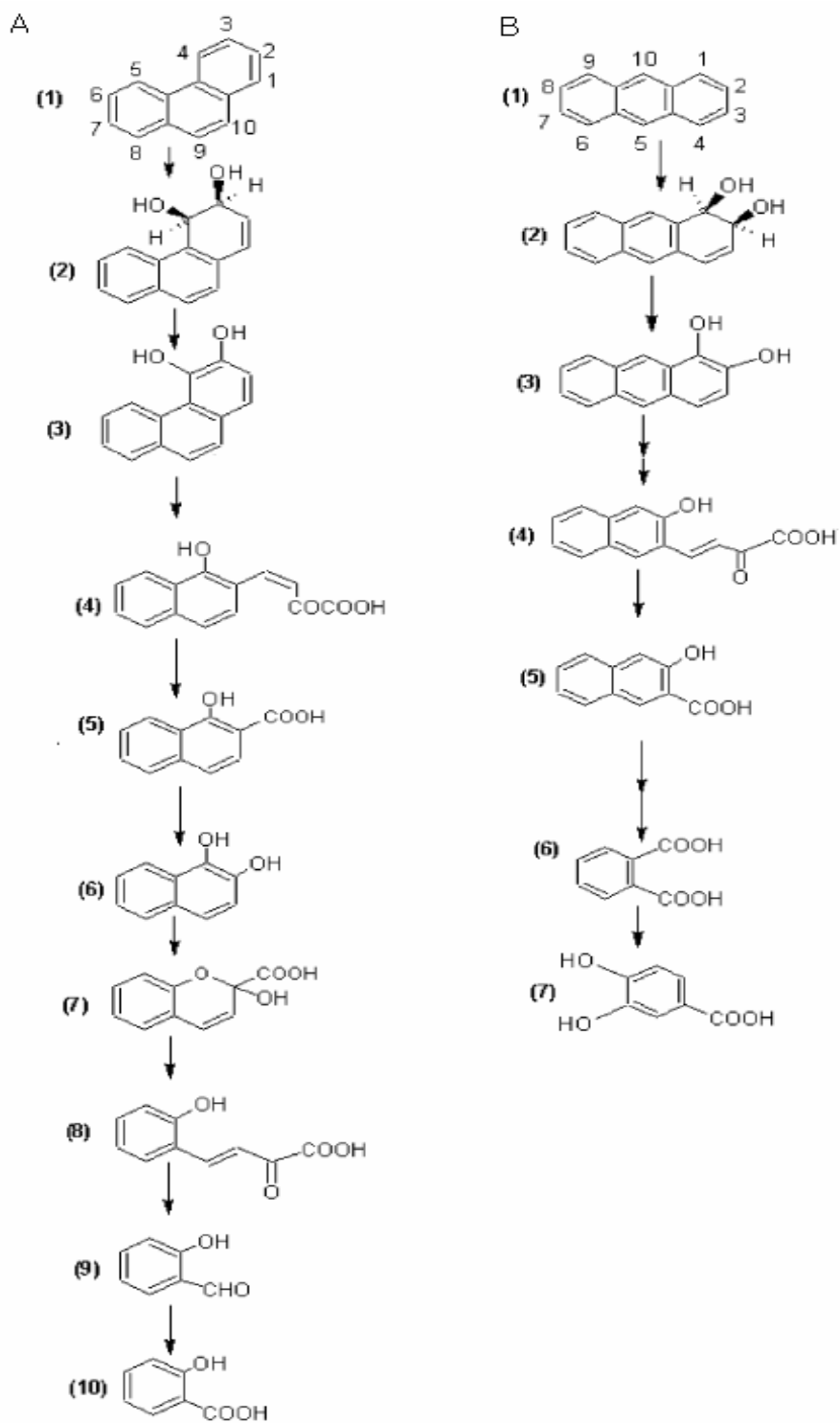


Fig. 1.6. Peripheral pathway for the catabolism of phenanthrene.

1.5. NDO in strains of *Pseudomonas*

The sequencing and characterisation of genes coding for enzymes involved in the catabolism of naphthalene was made possible by isolating the plasmid NAH7 (83 kb) from *P. putida* G7 [37]. The catabolic genes are organised in two operons: one codes for *upper pathway* enzymes that are involved in the conversion of naphthalene into salicylate, while the second codes for *lower pathway* enzymes that are involved in the conversion of salicylate into intermediates of the TCA cycle. Finally, a third gene (*nahR*), located between the two operons but transcribed independently, encodes the transcriptional activator (NahR), that acts for both [38]. NahR is necessary for the high level of *nah* gene expression and for their induction by salicylate [39].

Plasmids bearing the genes for the catabolism of naphthalene (called plasmid NAH), as well as derivatives of pWW60 from *P. putida* NCIB9816, pDTG1 from *P. putida* NCIB 9816-4 and pKA1 from *P. fluorescens* 5R, have been found to be very similar to the NAH7 plasmid of the G7 strain. The nucleotide sequences of genes coding for upper pathway enzymes of naphthalene, isolated from various strains of *Pseudomonas*, are available in the databases: *ndo* of *P. putida* NCIB 9816, *nah* of *P. putida* G7 and NCIB 9816-4, *dox* of *Pseudomonas* sp. C18, *pah* of *P. putida* OUS82 and *P. aeruginosa* PaK1 and *nah* of *P. stutzeri* AN10 [20]. The complete sequencing of these genes has been carried out for the strains OUS82, PaK1 and AN10; in other cases only partial sequencing was performed. The genetic organisation and sequencing of upper pathway catabolic genes of these strains is, however, similar to that of *nah* genes in the plasmid NAH7 of the G7 strain (Fig. 1.7).

In strains of *Pseudomonas*, the first four ORFs, *nahAaAbAcAd*, code for the four subunits NhaAa/Ab/Ac/Ad composed of NDO: NhaAa and NhaAb, an oxyreductase flavoprotein and a ferredoxin, provide a short electron transport system, formed by the oxidation of NAD(P)H. The two subunits NahAc and NahAd form the terminal hydroxylase complex (Fig. 1.8 a) in a $\alpha_3 \beta_3$ configuration (Fig. 1.8 b). The catalytic site, comprising a monoferric centre, is located inside the α subunit. The *nahB*, *nahF*, *nahC*, *nahE* and *nahD* genes, that code for the enzymes involved in the later breakdown stages of naphthalene, are found below the *nahAaAbAcAd* cluster [40] (Fig. 1.9).

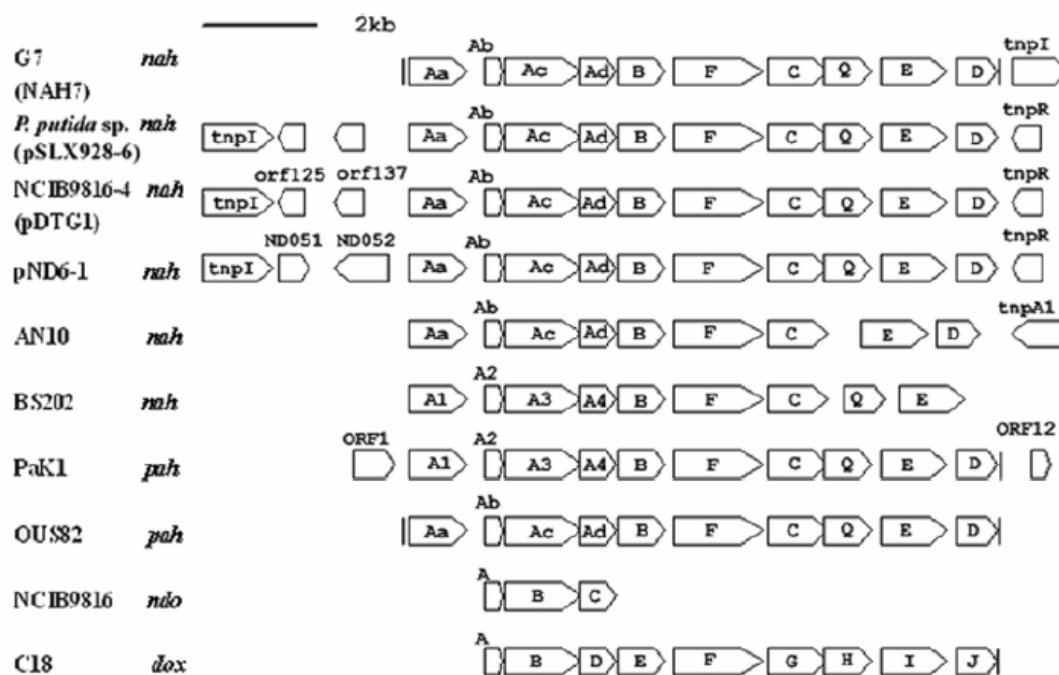
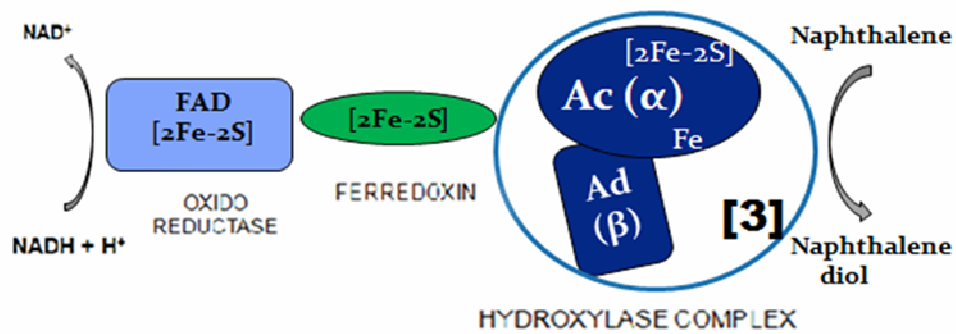


Fig.1.7. Catabolic genes of the upper pathway of naphthalene in strains of *Pseudomonas*.

Genes coding for the enzymes involved in the PAH breakdown pathways of the Pseudomonadaceae bacterial family are usually organised in clusters and, in a few species, have already been isolated and cloned. The analysis of these sequences has allowed the identification of varying portions and highly conserved portions, the latter usually being located on the regions coding for the enzyme's active site. These conserved portions are useful because they can be used to design probes that can look for new oxygenases. Research on new naphthalene dioxygenase is particularly interesting because it has been observed that enzymes with small differences in amino acid sequences have different substrate specificities.

a



b

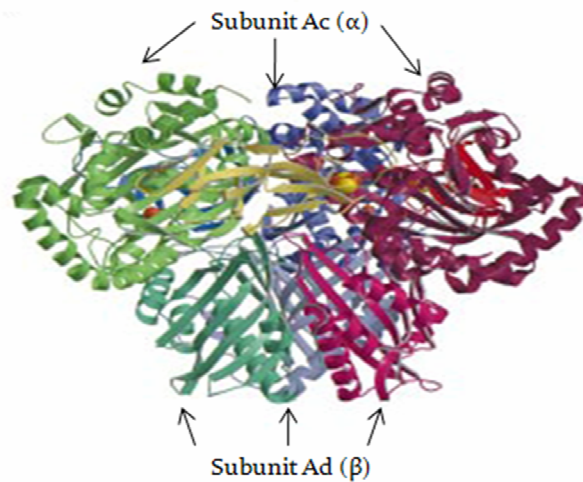


Fig. 1.8. a) Schematic representation of NDO enzymatic complex . b) crystallographic structure of NDO hydroxylase

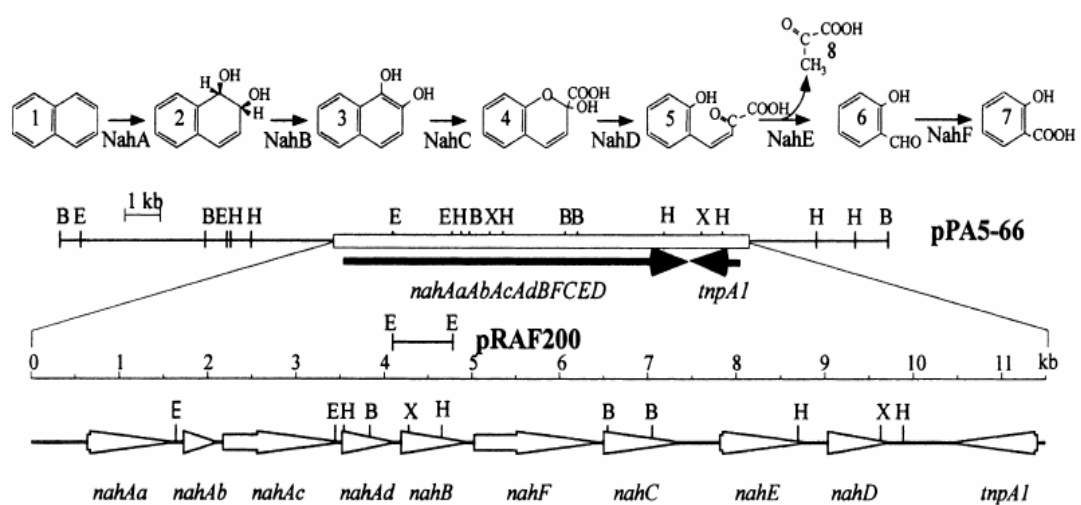


Fig. 1.9. Genetic organisation of the *nah* chromosome region of *P. stutzeri* AN10.

1.6. Sphingomonadaceae

The breakdown of aromatic molecules often occurs due to an interaction between different microbial strains through the formation of real microbial consortia in which, by establishing synergistic relationships, each different member is involved in only one, or a few, stages of the catabolic process [41]. A number of strategies can promote the breakdown of recalcitrant compounds such as the formation of a biofilm, a highly organised biological system in which the bacterial cells, in sessile form, are organised into a functional community with a specific spatial architecture and strong adherence to the substrate [42]. A further strategy is the production and release of biosurfactants, surfactants that are able to increase the bioavailability of highly lipophilic substrates [43].

Members of the Sphingomonadaceae family, that have recently been isolated and show a certain level of genetic richness, are capable of metabolising even very heterogeneous mixtures of mono- and polyaromatic compounds. Some authors argue that Sphingomonadaceae has such a broad metabolic versatility because the genes involved in the early phases of PAH breakdown are not combined in a single catabolic operon, but are scattered throughout the genome, a feature that allows them to easily reorganise their enzyme system. This alternative organisation of genes involved in the breakdown pathways of aromatic compounds distinguishes them from other bacterial genera, such as Pseudomonadaceae, in which the genes coding for breakdown enzymes tend to be grouped into operon units [44]. Based on phylogenetic analysis, that includes the 16S rDNA test and chemotaxonomic analysis, the Sphingomonadaceae were divided into four genera: *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* [45]. A fifth genus, *Sphingosinicella*, was added only later [46]. Bacteria belonging to this family are part of the subgroup α -Proteobacteria, are Gram negative, aerobic and chemotrophic, and their principle characteristic is the presence a particular class of molecule, the glycosphingolipids (GSL) in the external membrane. GSLs occupy the same position as lipopolysaccharides (LPS) in other Gram negative bacteria and perform much the same function, as well as exhibiting a similar structure to that of biosurfactants. The carbohydrate chain of GSL is, however, shorter than that of LPS, making the cell membrane of these bacteria more hydrophobic [45]; many Sphingomonadaceae are also able to secrete anionic heteropolysaccharides, known as *sphingans*.

1.6.1. Genes and enzymes for the catabolism of PAHs in Sphingomonadaceae

Members of the Sphingomonadaceae family are able to use a wide variety of aromatic compounds, including PAHs, as the sole source of carbon and energy. For example, *Novosphingobium aromaticivorans* F199, is able to break down toluene, xylene, p-cresol, biphenyl, naphthalene, dibenzothiophene, fluorene, salicylate e benzoate [47], while *Sphingobium yanoikuyae* B1 is able to grow on 1,2,4-trimethylbenzene, toluene, p-ethyltoluene, m- e p- xylene, biphenyl, naphthalene, phenanthrene and anthracene [48]. The ability of these microorganisms to breakdown polycyclic aromatic hydrocarbons is currently being studied in order to explain how these bacteria can use such a wide range of compounds.

The catabolic genes identified in the genus *Pseudomonas* can be located at the chromosome or plasmid level but, regardless of its position on the genome, they are grouped in clusters [44]. In contrast, the catabolic genes in Sphingomonadaceae are not grouped into operon units, but are scattered throughout the genome [49]. Some authors support the hypothesis that these catabolic sequences encountered a certain amount of evolutionary restriction that reduced the genetic exchange between Sphingomonadaceae and other bacterial genera [44].

The sequencing of the catabolic plasmid pNL1 (184 kb) from *N. aromaticivorans* F199 [50] has identified at least 79 genes grouped into 15 clusters, that code for the enzymes associated with the breakdown and transport of aromatic compounds. In respect of the oxygenase involved in the first step of the catabolism of phenanthrene, there appears to be a common gene arrangement in which the catalytic components (*bphA1a-f* and *bphA2a-f*) appear to interact with a single set of electron transporters (*bphA3* and *bphA4*). Many parts of the DNA sequence in the pNL1 regions coding for genes that are responsible for the catabolism of aromatic are similar to those of *S. yanoikuyae* B1, *S. paucimobilis* Q1, *Sphingomonas* sp. HV3, *Sphingomonas* sp. DJ77, *S. paucimobilis* TNE12 e *Sphingobium* sp. P2 [51]. These findings have suggested that an unusual arrangement of different genes, belonging to different catabolic pathways, is characteristic of the Sphingomonadaceae and that such organisation could contribute to their high versatility. In fact, if the amino acid similarity of the enzymes involved in the different catabolic pathways of aromatic compounds and the different organisation of the corresponding gene sequences are

considered, it's possible to hypothesise that the various gene clusters can modify their position and orientation within the genome, combining themselves into modules that can be added to other oxygenase genes [32]. Although many functional analyses on gene expression for the breakdown of aromatics have been reported, the function of each original dioxygenase remains unclear [52].

1.7. Bacterial metabolism of toluene

The breakdown of toluene can occur through dioxygen reactions (*via* TOD), through the monooxygenation of the aromatic ring in various locations [53, 54, 55], or through the progressive oxidation of the methyl group (*via* TOL) [56].

In *Pseudomonas putida* F1, the breakdown of toluene starts with the oxidation of the benzene ring by a toluene dioxygenase (TDO) forming the intermediate *cis*-dihydrodiol, followed by dehydrogenation that produces *d*-3-methylcatechol (Fig. 1.10), which then opens in the *meta* position [57, 58]. The genes that code for this enzyme form part of the *tod* operon that contains the genes for toluene dioxygenase (*tod* C1C2BA). In *Burkholderia cepacia* G4 and *Ralstonia pickettii* PKO1, toluene 2-monooxygenase and toluene 3-monooxygenase catalyse the formation of 3- methylcatechol through two, subsequent, monooxygenation reactions; the first of which produces the phenolic, *o*-cresol and *m*-cresol intermediates, respectively [53, 55] (Fig. 1.10). In *Pseudomonas mendocina* KR1, the *upper pathway* for the breakdown of toluene, that combines the hydroxylation of the aromatic ring and the oxidation of the methyl group, involves toluene-4-monooxygenase (T4MO) [59]. T4MO converts the toluene to *p*-cresol through a monooxygenation reaction. *p*-cresol is then oxidised, through subsequent transformations of the methyl group, to protocatechuic acid (the central intermediate of aromatic compound breakdown, together with catechol) that is finally mineralised *via* the *orto-pathway* (Fig. 1.11). In this particular case, given that it doesn't form catechol as the central intermediate, the aromatic ring opening involves another enzyme from the intradiolic dioxygenase family, the protocatechuate 3,4-dioxygenase. The dehydroxylated compounds produced by the reactions in the *upper pathway* are then broken down into intermediates that enter the Krebs cycle through the *lower pathway*.

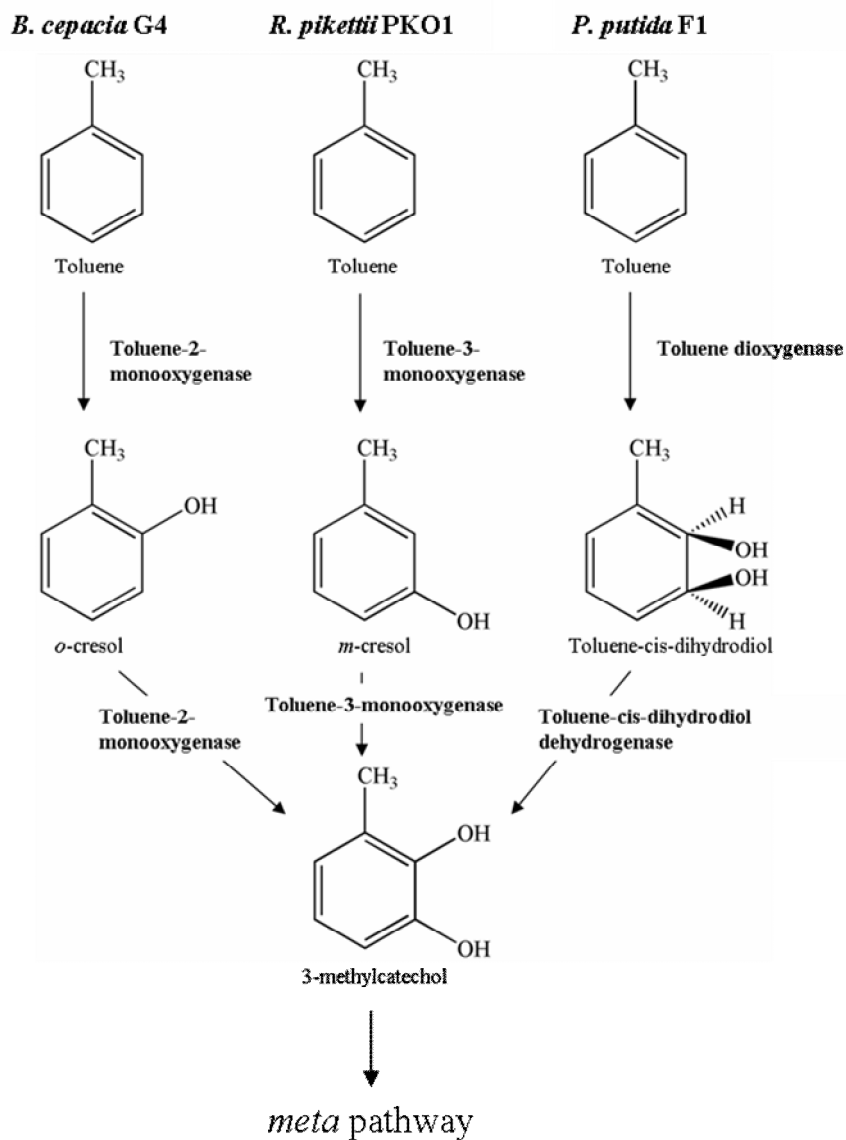


Fig. 1.10. Upper pathways for the breakdown of toluene in three different species *Pseudomonas*.

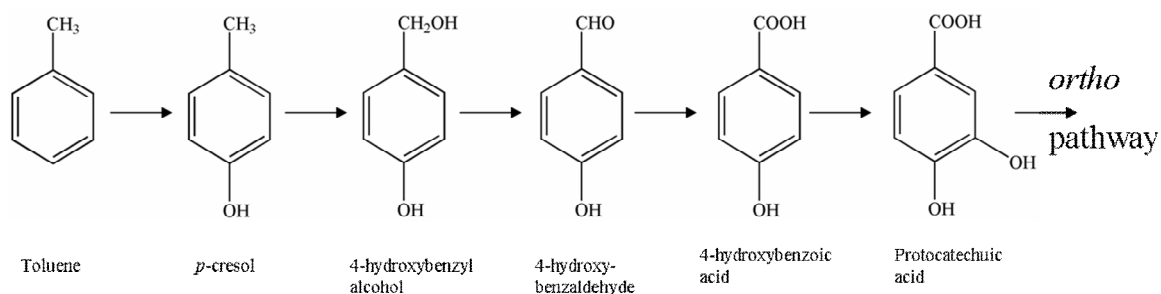


Fig. 1.11. Metabolism of toluene in *Pseudomonas mendocina* KR1.

1.7.1. Toluene/*o*-xylene monooxygenase in *Pseudomonas* sp. OX1

Pseudomonas sp. OX1 is one of the few microorganisms capable of breaking down toluene and *o*-xylene: this ability is due to the presence of an enzyme called toluene / *o*-xylene monooxygenase (ToMO). ToMO hydroxylates toluene and *o*-xylene, producing the corresponding phenols and is also responsible for the further hydroxylation of the phenolic intermediates in (di)methylcatechol [60]. Another characteristic of this bacteria, that contributes to its ability to adapt to polluted environments, is its resistance to organic and inorganic compounds containing mercury. The resistance to mercury is encoded by a plasmid, while the genes involved in the catabolism of hydrocarbons are located on the chromosome [61].

In the genome of *Pseudomonas* sp. OX1, the genes that code for toluene and *o*-xylene catabolic enzymes are organised into two operons: the first, called *tou operon*, codes for the ToMO complex; the second, called *phe operon*, contains the genes that code for Phenol Hydroxylase (PH), which is involved in the metabolism of phenol, and the genes coding for the enzymes of the *lower pathway*. The entire *upper pathway* includes, not only ToMO activity, but also that of PH. The breakdown process starts with the conversion of hydrocarbon into phenol, catalysed by ToMO. This event leads to the production of a mixture of *o*- *m*- and *p*-cresol from toluene and of 2,3- and 3,4-dimethylphenol from *o*-xylene; these intermediates are subsequently converted into 3,4-dimethylcatechol and 4,5-dimethylcatechol during a second monooxygenation reaction, catalysed by ToMO or PH (Fig.1.12). The breakdown starts, therefore, with two successive monooxygenation reactions, the first of which, catalysed by ToMO is not regiospecific, while the second, catalysed by ToMO or by PH, always occurs on a carbon in the *ortho* position in respect of the hydroxylated carbon [60]. The catechols produced by the monooxygenation reactions in the *upper pathway* are then broken down into intermediates that enter the Krebs cycle through the *lower pathway*.

The locus encoding ToMO maps in a 6 kb region of the *Pseudomonas* sp. OX1 chromosome, known as the *tou* (*toluene o*-*xylene utilisation*) locus: sequence analysis revealed the presence of a cluster of six ORFs called *touABCDEF*, each one coding for one of the subunits TouABCDEF which, together, constitute the whole enzyme complex (Fig. 1.13 a) [62].

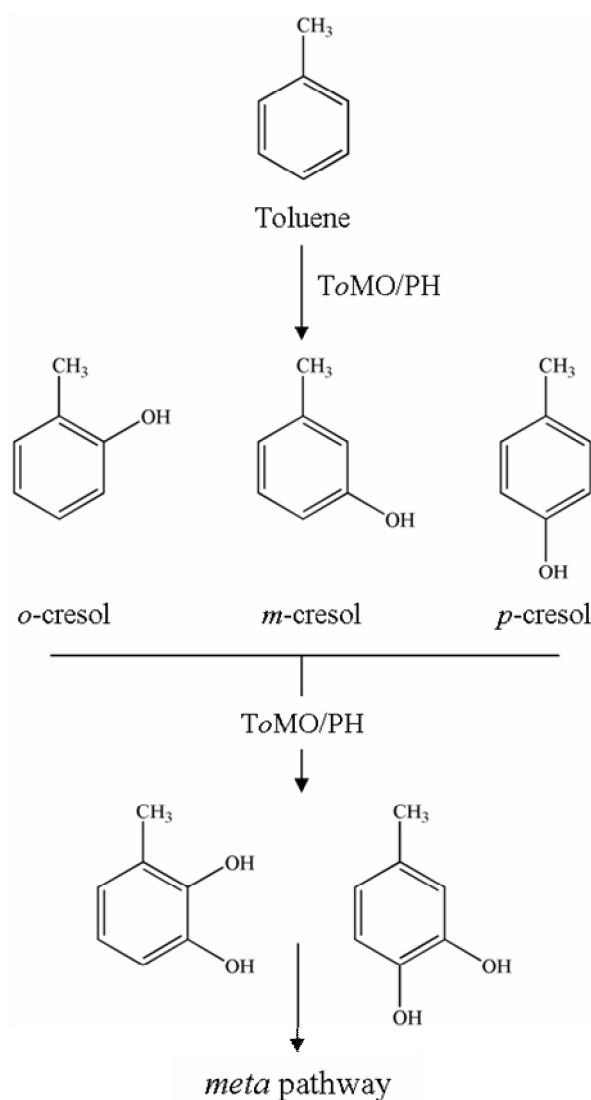


Fig. 1.12. Breakdown of toluene and *o*-xylene in *Pseudomonas* sp. OX1.

ToMO is composed of a flavoprotein oxyreductase (Tou F), a ferredoxin *Rieske-type* (Tou C), a terminal hydroxylase complex (ToMO-H) whose formation combines the subunits Tou A, B and E in configuration ($\alpha\beta\gamma$)₂ (Fig. 1.14), and a catalytic effector (Tou D) (Fig. 1.13 b) [63]. The electrons, indispensable for the catalysis, derived from the oxidation of NAD(P)H and, through a short, two-component transport system (Tou F and Tou C), converge towards the Tou A subunit in which there is a Fe-O-Fe diferric centre, the active site of the enzyme. The Tou D subunit is thought to carry out a regulatory function by interaction directly with the catalytic subunit Tou A, thereby influencing the speed of the enzyme's reaction [63].

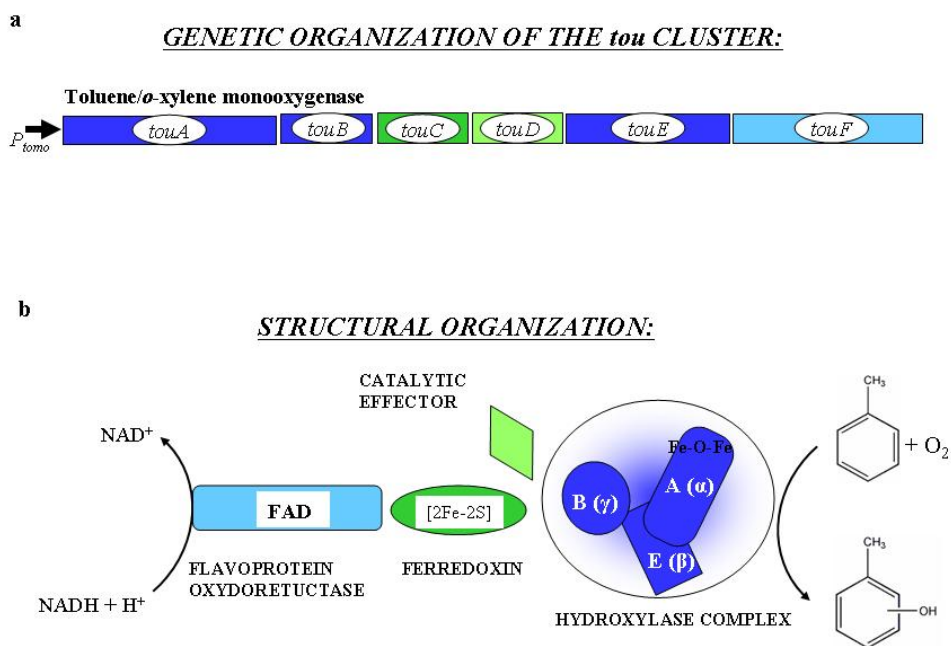


Fig. 1.13. a) Genetic organisation of the gene cluster encoding ToMO. b) Schematic representation of ToMO subunit organisation

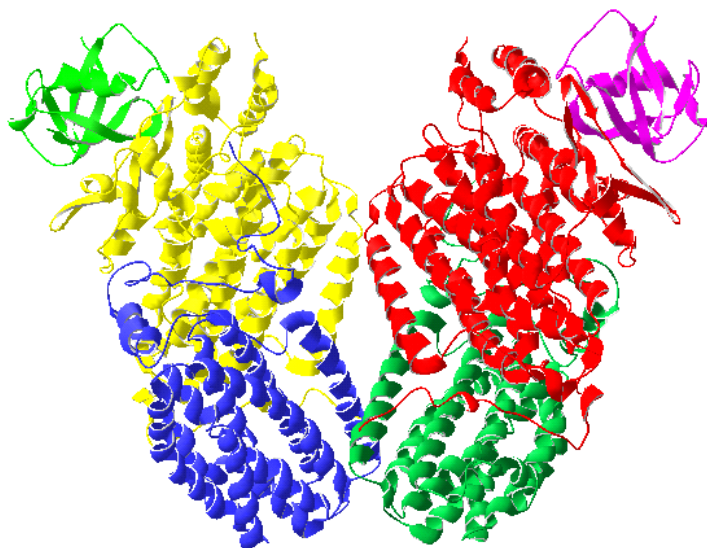


Fig. 1.14. Ribbon representation of ToMO-H (crystallographic data).

1.8. The contribution of molecular techniques and bioinformatics to the improvement of oxygenase systems

With modern genetic engineering, in a relatively short time you can create more stable and efficient enzymes that can be used by microorganisms for the purposes of degrading otherwise difficult compounds or for the production of intermediates of biotechnological interest. Several methods, including the construction of chimerical enzymes [64, 65] and random mutagenesis [66, 67] have been used by molecular biologists for the improvement of oxygenase systems.

These technologies, already in themselves advanced, are today of great support to bioinformatics; some resources, such as the NCBI, ExPASy and PDB portals, are available on the web and allow operations to be performed directly on-line starting from a simple consultation with a research database and comparison of nucleotide or protein sequences. These indispensable tools are accompanied by much open source software specifically designed for the study of various types of molecule; software dedicated to 3D structural analysis, such as PyMol and SPDBV [68], and those dedicated to the study of intermolecular interactions, for example, Autodock 4.0 [69] are particularly useful for the study of enzymes. This latter software is capable of automatically performing docking simulations between a small compound and a candidate macromolecule, DNA, RNA or protein, where a crystallographic structure exists. On the basis of data obtained from *in silico* structural analysis conducted on ToMO-H [70], from automated docking simulations and from information obtained from the current literature regarding the TouA E214G mutant [71], two new TouA mutants, D211A and D211A/E214G, were created through site-specific mutagenesis by the laboratory of Environmental and Molecular Microbiology in the Department of Structural and Functional Biology at the University of Insubria - Varese [72]. These mutants were used in biotransformation tests on toluene and nitrobenzene for a preliminary functional characterisation. Both mutants proved to be less efficient than the wild type enzyme in the biotransformation of toluene.

1.9. Biotransformation in industrial processes

Biotransformation is a reaction accelerated by biological catalysts (biocatalysts), in most cases isolated enzymes or whole cells [73, 74]. In the production of fine chemicals, for some time the advantage of using purified enzymes as heterogeneous catalysts has been well understood. In a few cases, however, the use of purified proteins for bio-industrial applications suffers from problems of costs connected with their purification, as well as from the fact that, for oligomeric molecules, the purification of functional molecules is not always possible. This is particularly true for oxygenase enzymes where the use of whole cells is more economical both in terms of time and cost [11]. Enzymes are protected inside the cell and, therefore, exhibit a longer half-life. In addition, the reactions catalysed by oxygenase require, usually, a certain number of cofactors that become difficult to provide *in vitro* but that, on the contrary, the whole cells provide naturally. In general it is simpler and less costly to generate these cofactors in metabolically active cells [75].

Both isolated enzymes and whole cells are currently used in industrial synthesis processes and represent an area of ongoing research [76].

The catalytic activity exploited to achieve biotransformation is normally released by the normal physiological activity of the enzyme and is also used on substrates that are structurally very diverse from those found in nature and in conventional cell metabolism. Many enzymes have a broad substrate specificity and modern protein engineering techniques allow a further widening of the range of biotransformable molecules [77].

Bioconversion techniques can offer a number of advantages that make them interesting as a complement to conventional synthesis techniques [78], in particular: high reaction selectivity (chemo-, regio-, stereoselectivity); mild and environmentally compatible reaction conditions; and reduced costs. Disadvantages, which often represent the main limitations of using biotransformations are: low stability of many biocatalysts in operational conditions; low yields; low numbers of commercially available biocatalysts; and a high development time for the process on an industrial scale. A few of the above stated advantages, particularly those concerning the different forms of selectivity, are only potential advantages, and are not always guaranteed. Enzyme catalysis can be extremely stereoselective but, when using substrates that differ from natural ones, selectivity may be only partial. On the other hand, some of the disadvantages normally associated with biotransformation seem, nowadays, to be largely outdated. The selectivity, stability and activity of many biocatalysts in particular reaction conditions can now be improved

through a variety of techniques such as: the selection of new enzymes through innovative screening techniques; improvement of the molecular biocatalysts using protein or metabolic engineering techniques; enzyme immobilisation; and finding the best reaction conditions (reaction engineering).

The production of molecules as single enantiomers or diastereoisomers is of growing importance, not only in the field of pharmaceuticals, but also in sectors of fine chemistry, food and materials [79]. The agencies that regulate the commercialisation of drugs now require separate toxicological studies for every impurity present in concentrations greater than 1%, including potential enantiomers or diastereoisomers of the active ingredient. It is therefore necessary to have available simple stereoselective methods (such as biotransformations) that allow enantiopure molecules to be obtained in limited quantities.

1.10. The potential of oxygenases in bioconversions

The hydroxylation reaction of natural aromatic compounds, catalysed by enzymes with oxygenase activity, has been identified as “potentially the most useful of all the biotransformations” [15]. Chemical processes that involve the introduction of an oxygen atom are often non-specific and at times require expensive and toxic reagents [80]; conversely, biocatalytic processes involving the use of oxygenase, in general, require milder reaction conditions.

The unique catalytic properties of oxygenases, such as the ability to hydroxylate unactivated carbon atoms in a regiospecific and/or enantiospecific way, gives these enzymes an indisputable value in the field of biosynthesis [75, 81]. Their ability to hydroxylate a number of hydrocarbons in specific positions and to generate specific enantiomers, also makes them useful for the biotransformation of compounds of pharmaceutical interest [11, 14]. In fact, aromatic di- and tri-hydroxylated compounds find applications in various fields such as the pharmaceutical industry, cosmetics and food; this is demonstrated, for example, by the global production of catechol, resorcinol, and hydroquinone, that reach levels estimated to be around 110,000 tonnes per year [82].

An example of a monooxygenase proposed as a biocatalyst for the synthesis of chiral epoxides is the styrene monooxygenase of *P. fluorescens*. These chiral compounds represent the starting molecules for the synthesis of a large number of fine chemicals [83]. Recently, site directed mutagenesis experiments were performed on the ToMO of *P. sp.*

OX1 to make it usable in the synthesis of high value compounds such as 4-methylresorcinol, methylhydroquinone, and pyrogallol [84]. One example, however, of a bacterial strain used as a breakdown biocatalyst is *Pseudomonas putida* ATCC 33015, a microorganism that is able to use *p*-xylene as the sole source of carbon and energy [85]. The ability of this bacterium has been studied in detail on the compound 2,5-dimethylpyrazine, where the oxidation product is an intermediate of important pharmaceutical synthesis processes; it is currently used in large-scale fermenters[11].

Even dioxygenase is used in biocatalysis. Toluene dioxygenase (TDO) is one such example: this enzyme is able to oxidise β -bromoethylbenzene leading to the formation of the corresponding *cis*-dihydrodiol, which is an important intermediate in the synthesis of codeine [86]. TDO is also used in the synthesis of Crixivan (Indinavir), an inhibitor of HIV protease. The key intermediate in the synthesis of this molecule is (-)-*cis*-(1*S*,2*R*)-1-aminoindan-2-ol; this compound can be synthesised directly from the enantiopure *cis*-(1*S*,2*R*)-dihydroxyindan, produced by TDO from indene. Another important biotransformation product, that has a broad use as a dye in the textile industry, is indigo, which is synthesised from indole; this substrate, recognised even by NDO, is oxidised to indoxyl, which demerises spontaneously to form indigo.

1.11. Metagenomic techniques for isolation of bacterial catabolic enzymes: the SIGEX method

Currently, research on genes coding for enzymes involved in the catabolism of PAH is based almost exclusively on the construction of genome libraries, followed by long and laborious screening and sequencing procedures. In recent years, as well as the genome of the individual microorganism, the attention of many research groups has focused on the “genome” of entire microbial communities (metagenomics) present in the environment being studied such as, for example, soil and water contaminated with hydrocarbons. Therefore, new techniques have been developed that can quickly identify, within a metagenomic library, catabolic genes that encode regulatory and structural elements.

One of the most advanced screening techniques of metagenomic libraries is based on the SIGEX (Substrate Induced Gene Expression) method [87]. This method exploits the fact that in bacteria many of the catabolic pathways present an operon-type genetic

organisation, which sees regulatory genes topologically close to structural genes. Using, for the construction of the library, an “operon trap” cloning vector, which contains, under the polylinker, a gene reporter that encodes a fluorophore, it is possible to check whether a particular substrate is capable of acting as an inducer, i.e. promoting the transcription of the insert; if there is evidence of transcriptional activity, there would be a good chance that the clone in question contains a substrate-dependent regulatory element. For the selection and collection of cells following induction, flow-cytometry techniques are used – this is another peculiarity of this method as it is typically used for the analysis of eukaryotic cells. The SIGEX protocol is based on four fundamental steps (Fig. 1.15): 1) construction of the metagenomic library using an “operon trap” vector containing a gene reporter (GFP); 2) treatment of the genomic library with the substrate of interest, for example a hydrocarbon belonging to the class PAH; 3) selection of cells that show fluorescence using flow-cytometry analysis; 4) isolation of GFP positive clones and sequencing of the fragments cloned in the relative constructs. The use of this technique allows, in a short time, clones containing substrate-dependent regulatory genes to be isolated. These elements can act as regulators of structural genes coding for enzymes that are involved in metabolism of the substrate inducer, or for enzymes responsible for the biosynthesis of useful breakdown molecules, for example, biosurfactants (bioemulsifiers). This powerful screening technique is perfectly suited to the research of genes encoding new bacterial oxygenase, as many of them have an operon-type genetic configuration (e.g. *ToMO*).

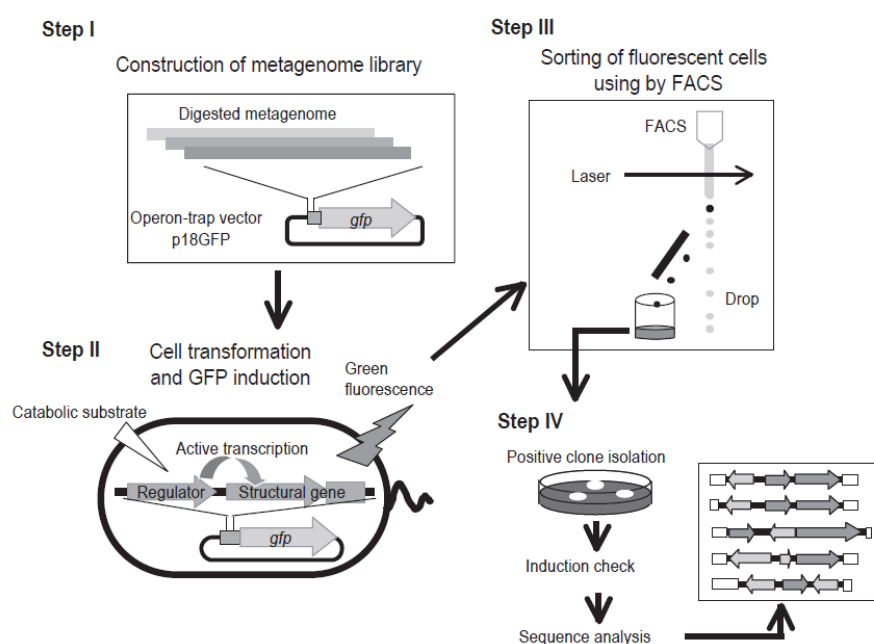


Fig. 1.15. Schematic representation of the SIGEX method.

2. Aims

Bacterial oxygenases are responsible for the activation of aromatic and aliphatic hydrocarbons by hydroxylation, which is the first step in their degradative pathways. These enzymes, due to their involvement in a variety of biocatalytic processes, are of great environmental and biotechnological interest.

The aims of this study include:

- 1) cloning new bacterial oxygenases using conventional culture methods (enrichment cultures prepared with PHAs) and developing new cloning techniques based on genomic library screening;
- 2) using biotransformation assays to carry out functional characterisations of the newly cloned enzymes, ToMO and the TouA D211A and D211A/E214G mutants, using, as substrates, compounds for which the activity of these enzymes has never previously been described and whose hydroxylated derivatives (1,2,3-trimethoxybenzene, anisole, benzophenone, bibenzyl, biphenyl, quinoline and *trans*-stilbene) are of great environmental, industrial and pharmacological interest.

In addition, with a view to achieving these objectives, it was resolved to standardise a biotransformation protocol, that produced good reaction yields for each substrate. Indeed, this condition was necessary in order to facilitate both qualitative and quantitative analyses.

3. Materials and Methods

3.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are reported in Tables 3.1 and 3.2 respectively. *E.coli* JM109 [88] was routinely used for plasmid construction and selection. Plasmids were isolated using standard procedures [89] or by use of purification kits (QUIAGEN) and introduced into the bacterial host by electroporation [90].

Table 3.1. Bacterial strains.

Strain	Features	Reference
<i>Pseudomonas</i> sp. N1	Able to use naphthalene as the sole carbon and energy source	This study
<i>Pseudomonas</i> sp. N2	Able to use naphthalene as the sole carbon and energy source	This study
<i>Sphingobium</i> sp. PhS	Able to use phenanthrene as the sole carbon and energy source	[91]
<i>E.coli</i> JM109	recA1, endA1, gyrA96, thi, hsdR17[rk+ mk+], supE44, relA1 λ - Δ [lac-proAB], F' [traD36, proAB+, lacIqZ Δ M15]	[88]
<i>E. coli</i> ER2566	F- λ - fhuA2 [lon] ompT lacZ::T7 gene 1 gal sulA11 Δ (mcrC-mrr)114::IS10 R(mcr-73::miniTn10-TetS)2 R(zgb-210::Tn10)(TetS) endA1 [dcm]	IMPACT™-CN, N.E. BioLabs, Inc

3.2. Culture methodology

3.2.1. Bacterial strains isolated from enrichment cultures

Bacterial strains were grown in Luria Broth (LB) [89] or M9 mineral medium (M9) [92] at 30 °C. In addition, M9 minimum medium, an M9/glucose solution 0.2% (w/v), was also used.

Biphasic system: the biphasic system consisted of M9 mineral medium as the water phase, and Dibutyl Phthalate (DBP) as the organic phase. The appropriate carbon source was added to DBP at a concentration of 400 mg/l; cultures were prepared by adding cells previously grown in M9 minimum medium to a mixture composed of 10% substrate/DBP solution and 90% M9, and incubated at 30 °C. The increase in biomass was evaluated using spectrophotometer readings at a wavelength of 600 nm.

Table 3.2. Plasmids.

Plasmid	Features	Reference
pGEM-TEasy	Ap ^r ; cloning vector	Promega
pGEM-3Z	Ap ^r ; cloning vector	Promega
pET-30° (+)	Km ^r ; expression vector	Novagen
pSTV28	Cm ^r ; cloning vector	Takara Biotechnology
pCZ2	Ap ^r ; pGEM-TEasy derivative containing NDO coding genes	This study
pCZ3	Ap ^r ; pGEM-TEasy derivative containing NDO coding genes	This study
pSG6	Ap ^r ; pGEM3Z derivative containing <i>bphA1fa2f</i> , <i>bphA3</i> and <i>bphA4</i> genes	[91]
pRO2	Ap ^r ; pGEM-3Z derivative containing <i>bphA1aA2a</i> , <i>bphA3</i> and <i>bphA4</i> genes	[93]
pNC4	Km ^r ; pET-30b derivative containing <i>bphA1aA2a</i> genes	[94]
pEM3	Cm ^r ; pSTV28 derivative containing <i>bphA3</i> and <i>bphA4</i> genes	[95]
pEM5	Cm ^r ; pSTV28 derivative containing <i>nahAaAb</i> genes	[95]
pEM6	Km ^r ; pET-30b derivative containing <i>bphA3</i> and <i>bphA4</i> genes	[95]
pEM7	Km ^r ; pET-30b derivative containing <i>nahAaAb</i> genes	[95]
pEM9	Cm ^r ; pSTV28 derivative containing RBS- <i>bphA1aA2a</i> genes	[95]
pEM10	Cm ^r ; pSTV28 derivative containing <i>bphA1aA2a</i> genes	[95]
pEM13	Km ^r ; pET-30b derivative containing RBS- <i>bphA3</i> and RBS- <i>bphA4</i> genes	[95]
pEM14	Cm ^r ; pSTV28 derivative containing <i>nahAcAd</i> genes	[95]
pEM15	Km ^r ; pET-30b derivative containing <i>nahAcAd</i> genes	[95]
pMZ1256	Ap ^r ; <i>tou</i> ABCDEF cloned in pGEM-3Z	[62]
pMZE214G	Ap ^r ; pMZ1256 derivative containing the TouA mutation E214G	[71, 72]
pMZD211A	Ap ^r ; pMZ1256 derivative containing the TouA mutation D211A	[72]
pMZD211A/E214G	Ap ^r ; pMZ1256 derivative containing the TouA mutation D211A/E214G	[72]

^a**Abbreviations:** Ap - Ampicillin; Km - Kanamycin; Cm - Chloramphenicol; RBS - Ribosome Binding Site.

Biofilm growth: 12-well plates were used in the biofilm growth assays. 100 µl of phenanthrene in ethanol solution (400 mg/l) was put into each well in order to obtain, after the evaporation of the solvent, a substrate film on the surface of each well. Subsequently, 1 ml of M9 was added to each well, which were each then inoculated with 10 µl of a cellular culture, prepared by diluting an M9 minimum medium culture, which had previously been incubated for 48 hours, to obtain an O.D. 600nm of 0.01. The quantity of biomass present in the supernatant was measured at 24-hour intervals using spectrophotometric analysis at a wavelength of 600 nm. The adherent biomass was then quantified using crystal violet staining and further spectrophotometric analysis: after removing the supernatant, an equal volume of a crystal violet solution (3 mg/ml) was added to each well and, after 20 minutes of incubation, the stain was removed by washing with a phosphate buffer (PB). Further to this, 1 ml of 96% ethanol was added to facilitate the detachment of the biofilm, which was then fragmented with a spatula. The suspension obtained was analysed using spectrophotometry at O.D._{600nm}. The biofilm index (BI) was calculated using the following formula:

$$\text{BI} = (\text{O.D.}_{600\text{nm}} \text{ of the adherent fraction } tX') \cdot (\text{O.D.}_{600\text{nm}} t0' / \text{O.D.}_{600\text{nm}} \text{ of the planktonic fraction } tX')$$

3.2.2. *E.coli* strains

Both *E. coli* JM109 and *E. coli* ER2566 were routinely grown in LB or M9 minimum medium at 37°C. Thiamine was added to M9 minimum medium at a concentration of 1 mM for the *E. coli* JM109 growth only. The antibiotics Ampicillin, Kanamycin and Chloramphenicol were added to the media, when necessary, at concentrations of 100 µg/ml, 50 µg/ml and 25 µg/ml, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (SIGMA-ALDRICH) was used as an inducer.

3.3. Methods of Molecular Biology

3.3.1. Cloning and DNA analysis

The genomic DNA and plasmid manipulation was carried out using commercial kits (QUIAGEN – SIGMA- ALDRICH). The PCR amplifications were performed using PCR Master Mix (FERMENTAS) and the thermocycler model PTC-100™ Programmable Thermal Controller (MJ Research). The primers used are detailed in Table 3.3 and the DNA control analyses were performed by agarose gel electrophoresis. The DNA sequencing was performed, externally, by *BMR Genomics*.

Table 3.3. Primers.

Primer	Sequence	Restriction site
16SF	5'-AGAGTTTGATCCTGGCTCAG-3'	
16SR	5'-CTACGGCTACCTTGTTACGA-3'	
NahAcF	5'-TGGCGATGAAGAACTTTTCC-3'	
NahAcR	5'-AACGTACGCTGAACCGAGTC-3'	
NahAabcdF	5'- GAGCT CATGGAATTCTCGTACTACCGA-3'	Sac I
NahAabcdR	5'- CTCGAGT CACATAAAGATCATCACATTGTGCGT-3'	Xho I
Ph321F	5'-TTCTCGGTCGGGACTTCCAA-3'	
Phn671R	5'-GGCAACCAGATCTGTCATG -3'	
Phn2F (<i>bphA1fa2f</i>)	5'-TTA AGCT TATGAATGGATCGGCGGCACTC -3'	Hind III
Phn2R	5'-TT GAGCT CCTTATACAAAGAAATAGAGATTCTTGTC-3'	Sac I
A1A2aF (<i>bphA1aA2a</i>)	5'-TTA AGCT TATGAATGGATCGGCGGCACTC -3'	Hind III
A1A2aR	5'-TT GAGCT CCTTATACAAAGAAATAGAGATTCTTGTC-3'	Sac I
FdF (<i>bphA3</i>)	5'- TTTCTAGA ATGTCAAACAAATTGCGACTCTGT-3'	Xba I
FdR	5'- TTGGTACCT CATGCTGCTCCTTCGGG-3'	Kpn I
FdRF (<i>bphA4</i>)	5'- TTGGTACCAT GAAATCGATAGCCGTGGTC-3'	Kpn I
FdRR	5'-TTA AGCTT CTAACCAGCCTGCTTGAGGATTTC-3'	Hind III

^a Sequences recognised by restriction endonucleases are in bold.

3.3.2. Protein analyses

The extraction of proteins from culture samples induced with IPTG was carried out in accordance with standard protocol, treating the cells with a lysis buffer and a cocktail of protease inhibitors (SIGMA-ALDRICH); the samples were then treated with sonication

and then centrifuged. The supernatant, containing the soluble protein, was separated from the pellet; the latter, containing the insoluble protein, was further treated with SDS sample buffer. The quantification of total protein was carried out by treating the samples with Bradford reagent (SIGMA-ALDRICH) and subsequent spectrophotometric analysis at 595 nm.

The SDS- Polyacrylamide Gel Electrophoresis (PAGE) technique [96] was used for the one-dimensional analysis of the proteins. An aqueous solution containing ethanol (25%), acetic acid (10%) and Coomassie brilliant blue R250 (2.5 g/l) was used for the staining; for removing the stain, an aqueous solution containing ethanol (25%) and acetic acid (8%) was used.

3.4. Biotransformations

3.4.1. Cellular growth and induction

Cells carrying the plasmids that express the enzyme of interest were grown overnight (o.n.) in 20 ml of the selective M9 minimum medium. An aliquot of overnight culture, sufficient to achieve an O.D._{600nm} of 0.1, was added to 250 ml of M9 minimum medium and incubated with agitation at 37 °C until reaching an O.D._{600nm} of 0.6. At this stage, IPTG was added to the culture at a concentration of 1 mM, and the induction level was checked at 15 minute intervals using the indigo test. On reaching the maximum level of induction (from 2 to 3 hours according to the enzyme used), the culture was centrifuged and the cells stored, in pellet-form, at -20 °C. The same protocol was carried out using LB medium; in this variant glucose was added only to the overnight growth phase in order to minimise the expression of the enzyme.

3.4.2. Chemicals

The compounds used as substrates in biotransformation, purchased from SIGMA-ALDRICH, are detailed in Table 3.4. The solid, water-insoluble molecules were used at a theoretical final concentration of 10 mM and administered in a cellular suspension in a pure form. Nitrobenzene, quinoline and indole, as liquids having a certain degree of solubility in water, were used at concentrations of 0.2, 1 and 2 mM respectively, and administered to the cellular suspension in pure form. Toluene, a volatile and poorly water-

soluble compound, was administered to the cellular suspension in a vapour phase in order to obtain a concentration in the aqueous phase of around 5 mM.

The mono- and dihydroxylated compounds, used as standards in both the qualitative and quantitative analyses (purchased from SIGMA-ALDRICH) are summarised in Table 3.5.

Table 3.4. Substrates used in the biotransformation assays.

Compound	Features
1,2,3-trimethoxybenzene	M. F.: C ₉ H ₁₂ O ₃ ; M. W.:168 g/mol; W. S.: insoluble; P. S. at 25 °C: solid.
Anisole	M. F.: C ₇ H ₈ O; M.W.: 108.14 g/mol; W.S.: 143 mg/l (soluble); P.S. at 25 °C: liquid.
Benzophenone	M. F.: C ₁₃ H ₁₀ O; M.W.:182.22 g/mol; W.S.: insoluble; P.S. at 25 °C: solid
Bibenzyl	M. F.: C ₁₄ H ₁₄ ; M. W.:182.26 g/mol. W. S.: insoluble; P. S. at 25 °C: solid.
Biphenyl	M. F.: C ₁₂ H ₁₀ ; M. W.: 154.21 g/mol. W. S.: insoluble; P. S. at 25 °C: solid.
Indole	M. F.: C ₈ H ₇ N; M. W.: 17.5 g/mol; W. S.: 1,90 g/l (soluble); P. S. at 25 °C: liquid.
Naphthalene	M. F.: C ₁₀ H ₈ ; M. W.: 128.17 g/mol; W. S.: insoluble; P. S. at 25 °C: solid.
Nitrobenzene	M. F.: C ₆ H ₅ NO ₂ ; M. W.: 123.11 g/mol; W. S.: 1,90 g/l; P. S. at 25 °C: liquid.
Phenanthrene	M. F.: C ₁₄ H ₁₀ ; M. W.: 178.23 g/mol; W. S.: insoluble; P. S. at 25 °C: solid.
Quinoline	M. F.: C ₉ H ₇ N; M. W.: 129.16 g/mol; W. S.: soluble; P. S. at 25 °C: liquid.
Toluene	M. F. : C ₇ H ₈ ; M. W.: 92.14 g/mol; W. S.: 0.47 g/l (insoluble); P. S. at 25 °C: liquid.
<i>Trans</i> -stilbene	M. F. : C ₁₄ H ₁₂ ; M. W.: 180.24 g/mol; W. S.: insoluble; P. S. at 25 °C: solid.

^a**Abbreviations:** M. F., Molecular Formula; M. W., Molecular Weight; W. S., Water Solubility; P. S., Physical State.

Table 3.5. Standards used for the quantification of biotransformations products

Compound	Features
2,6-dimethoxyphenol	M. F.:C ₈ H ₁₀ O ₃ ; M.W.: 154.16 g/mol.
2-hydroxybiphenyl	M. F.:C ₁₂ H ₁₀ O; M.W.: 170.21 g/mol.
2-hydroxyquinoline	M. F.:C ₉ H ₇ NO; M.W.: 170.21 g/mol.
2-nitrophenol	M. F.:C ₆ H ₆ NO ₃ ; M.W.: 139.11 g/mol.
3,4,5-trimethoxyphenol	M. F.:C ₉ H ₁₂ O ₄ ; M.W.: 184.19 g/mol.
3-nitrophenol	M. F.:C ₆ H ₆ NO ₃ ; M.W.: 139.11 g/mol.
4-hydroxybenzophenone	M. F.:C ₁₃ H ₁₀ O ₂ ; M.W.: 198.22 g/mol.
4-hydroxybiphenyl	M. F.:C ₁₂ H ₁₀ O; M.W.: 170.21 g/mol.
4-methoxyphenol	M. F.:C ₇ H ₈ O ₂ ; M.W.: 124.14 g/mol.
4-nitrophenol	M. F.:C ₆ H ₆ NO ₃ ; M.W.: 139.11 g/mol.
<i>m</i> -cresol	M. F.:C ₇ H ₈ O; M.W.: 108.14 g/mol.
N-oxoquinoline	M. F.:C ₉ H ₇ NO; M.W.: 145.16 g/mol.
<i>o</i> -cresol	M. F.:C ₇ H ₈ O; M.W.: 108.14 g/mol.
<i>p</i> -cresol	M. F.:C ₇ H ₈ O; M.W.: 108.14 g/mol.
Phenol	M. F.:C ₆ H ₆ O; M.W.: 94.11 g/mol.
α -naphthol	M. F.:C ₁₀ H ₈ O; M.W.: 144.2 g/mol.

^a**Abbreviations:** M. F., Molecular Formula; M. W., Molecular Weight.

3.4.3. The indigo test

The indigo test was performed by adding 40 μ l of a 50 mM indole solution in ethanol and water (1:1) to 1 ml of induced culture sample. The appearance of a blue precipitate was visually assessed by observing how much time passed between the treatment with indole to the appearance of the indigo colour.

3.4.4. Biotransformation assays

The induced cells were resuspended in M9 minimum medium in order to obtain a reaction volume of 10 – 20 ml with an O.D._{600nm} of 2.0. The substrate was added to the suspension in a manner and in amounts dependent on its physical and chemical properties

(see S. 3.3.2). In the case of toluene, a particular type of flask was used comprising two separate compartments, one containing the cellular suspension, the other for the toluene; the system was sealed to allow a saturated atmosphere to be obtained. The reaction mixture was incubated with agitation at 37 °C. At prescribed times, 1 ml samples were collected from the mixture and prepared for analysis.

3.4.5. Analytical techniques

Identification of biotransformation products

Biotransformation products were identified by GC-MS analysis (Thermo Scientific, series DSQ II (Dual Stage Quadrupole GC-MS)); a polysilphenylene-siloxane gas chromatography capillary column (s.n. 260F298P) was used to separate the molecules and helium was used as a gas carrier. The bioconversion product was introduced to the organic phase by extraction using ethyl acetate, at a ratio of 1:1; the organic phase was subsequently anhydrous with Na₂SO₄, centrifuged for 15 minutes at 5000 rpm and filtered using 20 µm syringe filters (MINISART RC 15). 1 µl of the sample was manually injected into the GC-MS and analysed using the preset FullScan. At the end of the analysis, the data were processed using Xcalibur software (THERMO SCIENTIFIC). The molecules present in the sample were identified by comparing their mass spectra with those in the library supplied with the equipment (NIST).

Quantification of biotransformation products

Quantification of biotransformation products was carried out using High Performance Liquid Chromatography (HPLC) reversed phase (equipment model Agilent 1100), coupled with UV-Visible spectrophotometry. The column model, ZORBAX ECLIPSE PLUS C18, was used for all the analyses. Elution mixtures were prepared using CHROMASOLV series reagents (SIGMA-ALDRICH) and MQ water produced by MILLIPORE equipment. The biotransformation products derived from water-soluble substrates were analysed in the aqueous phase after centrifugation and filtration of the collected sample. Biotransformation products of insoluble substrates were introduced to the organic phase by extraction performed in the same manner as the GC-MS analysis. The samples obtained from each biotransformation experiment were loaded into the column using a sampling loop with a 5 µl capacity. The elution of the samples was performed by the use of a methanol-H₂O

solution, in differing ratios according to the substrate analysed, at a flow rate of 0.5 ml/min. The data were processed using the supplied software.

4. Results

4.1. Looking for new oxygenases involved in aromatic compound breakdown

4.1.1. Analysis of a bacterial strain collection and cloning of NDO genes from *Pseudomonas* sp.

In looking for oxygenases that are potentially usable in biotransformation processes, several bacterial strains have been studied. Bacterial strains were isolated from anthracene, fluorene, naphthalene and phenanthrene enrichment cultures during a previous study [97].

Each bacterial strain's ability to grow in both planktonic and biofilm form was evaluated; for each strain, the isolation substrate was used as the sole carbon and energy source. The liquid cultures were prepared using a biphasic system (M9 minimum medium/dibutylphthalate) in which the substrate was dissolved in 100 mM dibutylphthalate (DBP) and the biomass increase was evaluated by spectrophotometry at 600 nm; Table 4.1. shows the qualitative growth results after 10 days of incubation: N1 and N2 strains grew quickly on naphthalene while I4, I8 and I15 strains showed poor growth on this substrate. For cultures on phenanthrene, A1 and F1 strains showed good growth and the other 14 strains showed poor growth. In the cultures prepared with anthracene and fluorene, no growth was observed.

The purity of the cultures was verified by plating them on LD agar medium; surprisingly, the A1 and F1 strains each showed two different types of colony morphology (A1a, A1b and F1a, F1b). Consequently, it was hypothesised that a bacterial synergy (consortium) was present in the cultures. To test this hypothesis, a liquid culture from each type of colony was independently prepared, although no growth was observed. Unexpectedly, the reassembled hypothetical consortium showed no growth either. To evaluate the presence of phenanthrene dioxygenase (PhDO), a peripheral enzyme frequently involved in the first steps of phenanthrene oxidation, a PCR analysis on the A1a, A1b, F1a and F1b strains was performed using the primers Ph321F and Phn671R. For the A1a strain, only one amplicon reportable to the major PhDO subunit was observed. However, sequence analysis (BLAST algorithm – www.ncbi.nlm.nih.gov) showed a very high similarity to a PhDO variant currently being studied in this laboratory. For this reason these four strains were not analysed further.

The 14 strains that showed poor growth in the presence of phenanthrene (Table 4.1) were, however, analysed. A biofilm growth system was prepared in order to verify each strain's ability to grow as a biofilm in the presence of phenanthrene.

Table 4.1. Bacterial strain collection growth tests performed using anthracene, fluorene, naphthalene, and phenanthrene, supplied as the sole carbon and energy source, in the biphasic system.

Strain	Substrates			
	Naphthalene	Phenanthrene	Anthracene	Fluorene
A1	-	+	-	-
A2	-	-	-	-
A3	-	-	-	-
F1	-	+	-	-
F2	-	-	-	-
F3	-	-	-	-
F4	-	-	-	-
FL1	-	-	-	-
FL2	-	-	-	-
FL3	-	-	-	-
FL4	-	-	-	-
I1	-	-	-	-
I2	-	-/+	-	-
I3	-	-	-	-
I4	-/+	-/+	-	-
I5	-	-	-	-
I6	-	-/+	-	-
I7	-	-/+	-	-
I8	-/+	-/+	-	-
I9	-	-/+	-	-
I10	-	-/+	-	-
I11	-	-/+	-	-
I12	-	-/+	-	-
I13	-	-/+	-	-
I14	-	-/+	-	-
I15	-/+	-/+	-	-
I16	-	-/+	-	-
I17	-	-/+	-	-
N1	+	-	-	-
N2	+	-	-	-

^a**Symbols:** +, good growth; -, no growth; +/-, poor growth; +*, mixed culture (consortium).

Among the mechanisms by which *in vivo* microorganisms can increase the bioavailability of hydrophobic substrates are biofilm formation and production of cell-bound emulsifiers [98]. A biofilm is an aggregate of microorganisms in which cells adhere to each other on a surface in response to various environmental inputs. These adherent cells are frequently embedded within a self-produced extracellular polysaccharide (EPS) matrix. The biofilm-forming ability of each strain was evaluated by the biofilm index (BI): the biofilm index was calculated 1) in the presence of the M9 minimal medium only (1st control), 2) in the

presence of a M9 minimal medium containing ethanol, which is the solvent used to dissolve the substrate for its adhesion to the plate surface (2nd control) and 3) in the presence of M9 minimal medium containing the substrate. For three strains only, I10, I12 and I15, a BI value (after 72 hours), apparently related to growth on phenanthrene film, was observed (App. 4.1), however, PCR analysis performed on these strains did not show any reportable PhDO signal.

All the performed analyses suggested that most of the tested strains were unable to grow using the supplied substrate as the sole carbon and energy source; however, they were highly tolerant of the enrichment substrates and probably able to grow due to other available cell-derived organic compounds. Accordingly, further investigations were focused on N1 and N2 strains, which are able to use naphthalene as the sole carbon and energy source.

4.1.1.1. Taxonomic characterisation of N1 and N2 strains

Initially, N1 and N2 strains were classified by conventional microbiological techniques: they were found to be gram-negative and catalase / cytochrome oxidase positive; both showed the same morphology (bacillus) and characteristics of the pseudomonadaceae family. In addition, a 16S rDNA analysis was performed on the two strains using 16SF and 16SR primers (App. 4.2). Using the BLAST algorithm the N1 and N2 16S rDNA sequences were aligned with databank sequences and the results confirmed that both the N1 and N2 strains are members of the Pseudomonadaceae family (Table 4.2)

4.1.1.2. Preliminary functional characterisation of N1 and N2 strains

N1 and N2 strains were grown in a biphasic system, in which naphthalene was supplied as the sole carbon and energy source. As expected, the cultures displayed a good growth after 24 h of incubation (Fig. 4.1a and b). On a solid naphthalene medium, both the N1 and N2 strains produced brown colonies after 10 days of incubation at 30°C.

To verify the presence of “peripheral” and “terminal” oxygenases in the N1 and N2 strains, two colorimetric tests were performed: the indole test, to evaluate the presence of peripheral oxygenase activity and the catechol test, to verify the presence of oxygenase enzymes able to open the aromatic ring in the extra-diol position.

Table 4.2. N1 and N2 16S rDNA alignment (BLAST).

Isolated	Accession	Description	Max score	Total score	Query coverage
N1	GU086437.1	Pseudomonas sp. M3 16S ribosomal RNA gene, partial sequence	2542	2542	100%
	EU344963.1	Pseudomonas sp. G5(2008) 16S ribosomal RNA gene, partial sequence	2542	2542	100%
	AY456708.1	Pseudomonas sp. PILH1 16S ribosomal RNA gene, partial sequence	2542	2542	100%
N2	EU727192.1	Pseudomonas sp. MT-I3 16S ribosomal RNA gene, partial sequence	2562	2562	100%
	AY762360.1	Pseudomonas sp. A 16S ribosomal RNA gene, partial sequence	2562	2562	100%
	DQ026293.1	Pseudomonas sp. M1 16S ribosomal RNA gene, partial sequence	2558	2558	100%

Many oxygenases are able to convert indole to indigo, which is easily detectable due to its blue colour. A biotransformation assay was performed by adding indole to N1 and N2 cultures grown on naphthalene only ($O.D._{600} = 0,6$). As a control, the same experiment was performed using cultures grown in the sole presence of glucose. After 30 minutes of incubation, indigo production was observed in the naphthalene culture while the control remained colourless. The catechol test was prepared in the same way. After a few minutes the culture grown in the presence of naphthalene turned yellow, the typical colour of hydroxymuconic semialdehyde, the product deriving from the aromatic ring opening in the extra-diol position. The control remained colourless.

These results suggest that N1 and N2 strains carry both (naphthalene-inducible) peripheral and terminal oxygenases.

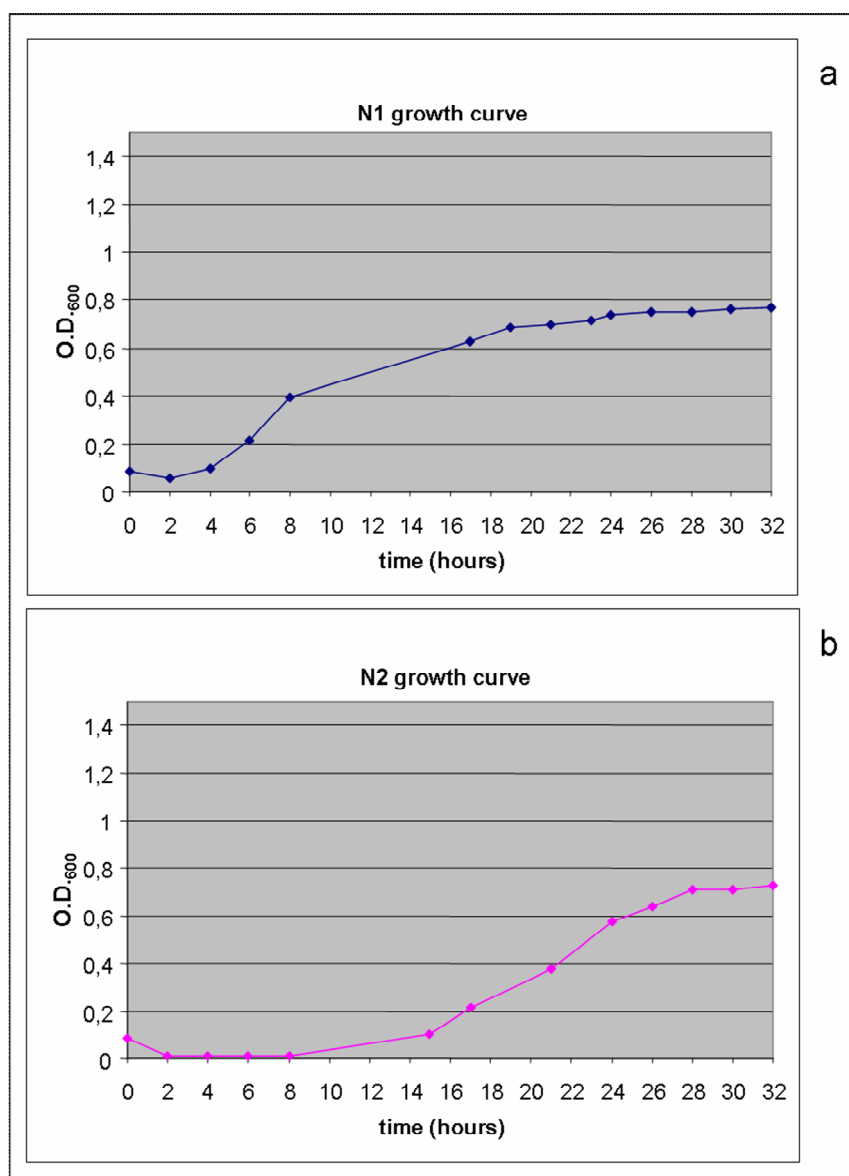


Fig. 4.1. N1 (a) and N2 (b) growth curves on naphthalene.

4.1.1.3. Cloning of NDO gene clusters from *Pseudomonas* sp. N1 and N2 strains

The preliminary approach to peripheral oxygenase identification was the amplification, by PCR, of the most conserved regions.

A molecular analysis, aimed at verifying the presence of naphthalene dioxygenase gene(s), was carried out on the N1 and N2 strains by a PCR reaction using NahAcF and NahAcR primers that were previously designed to amplify the *P. putida* G7 NDO major subunit (α) encoding gene [99]. The PCR reaction produced amplicons of the expected length (1000 bp) for the N1 strain only (App. 4.3).

The amplified DNA was sequenced and the results were compared with the databank sequences. The results showed a high similarity with certain NDO sequences isolated from various members of the pseudomonadaceae family (Table 4.3).

The PCR reaction was repeated on the N2 strain using two new primers designed on the most conserved part of the NDO α subunit sequence [100]. As a control, the N1 strain DNA was used. The PCR reaction produced the expected amplicon (136 bp) for both strains.

A new alignment with databank sequences showed that the N2 amplified DNA had high sequence similarity with certain pseudomonadaceae NDO sequences (Table 4.4)

Table 4.3. N1 *nahAc* sequence alignment.

Accession	Description	Max score	Total score	Query coverage
D84146.1	<i>Pseudomonas aeruginosa</i> pah genes for 12 ORFs (components of naphthalene dioxygenase, dehydrogenase, hydratase-aldolase, isomerase)	1640	1640	100%
AF039533.1	<i>Pseudomonas stutzeri</i> naphthalene degradation upper-pathway gene cluster (<i>nahAa</i> , <i>nahAb</i> , <i>nahAc</i> , <i>nahAd</i> , <i>nahB</i> , <i>nahF</i> , <i>nahC</i> , <i>nahE</i> , and <i>nahD</i>) and transposon-like protein (<i>tnpA1</i>) gene, complete cds	1580	1580	100%

Table 4.4. N2 *nahAc* sequence alignment.

Accession	Description	Max score	Total score	Query coverage
AM743147.1	Uncultured <i>Pseudomonas</i> sp. partial <i>pahd</i> gene for PAH-dioxygenase gene, clone Berre6A4B-6	132	132	98%
AY196829.1	<i>Pseudomonas stutzeri</i> isolate 67 naphthalene dioxygenase iron sulfur protein (<i>nahAc</i>) gene, partial cds	132	132	98%
AF306442.1	<i>Pseudomonas putida</i> 5IIANH naphthalene dioxygenase iron sulfur protein 2 (<i>nahAc2</i>) gene, partial cds	132	132	98%

From previous results it was supposed that both the N1 and N2 strains carried the entire NDO cluster. On the basis of the sequence analysis, two new primers were designed in order to amplify the first four ORFs encoding the three NDO components: Fe-S flavoreductase (*NahAa*); 2Fe-2FS ferredoxin (*NahAb*) and a hydroxylase complex $\alpha_3\beta_3$ (*NahAc/d*). The new primers, *NahAabcdF* and *NahAabcdR*, carrying *SacI* and *XhoI*

restriction sites at the 5' ends respectively, were designed on the basis of the sequence of the *Pseudomonas aeruginosa* NDO genes [40]. For both strains, the PCR reaction produced the expected amplicon of 3,5 kb. The PCR products were cloned in a pGEM-T easy-cloning vector used to transform the heterologous host *E. coli* JM109. The obtained construct is shown in Figure 4.2. Several positive clones were analysed by restriction analysis in order to verify the correct insert orientation: two clones, pCZ2 and pCZ3, from the N1 and N2 strains respectively, showed the expected restriction pattern and were selected for further analyses (Fig 4.3).

pCZ2 and pCZ3 DNA cloned fragments were sequenced and sequence analysis showed that the two fragments were identical. The analysis also confirmed the presence of the 4 ORFs encoding the three NDO components (Tab 4.5) and the sequences showed a nucleotidic similarity of 100%. Moreover, the cloned pCZ2 and pCZ3 sequences showed a high amino acid similarity (98-99%) with three sequences in the data bank (D84146.1, AF039533.1, HM204990.1 - www.ncbi.nlm.nih.gov) about which nothing has been published in the current literature. The amino acid similarity with the other sequences was similar or less than 85%.

The NDO encoding sequence from *Pseudomonas* sp. N1 was deposited in GeneBank (access: HM368649). To perform the biotransformation assays (described below) *E. coli* JM109 carrying the pCZ2 plasmid was used.

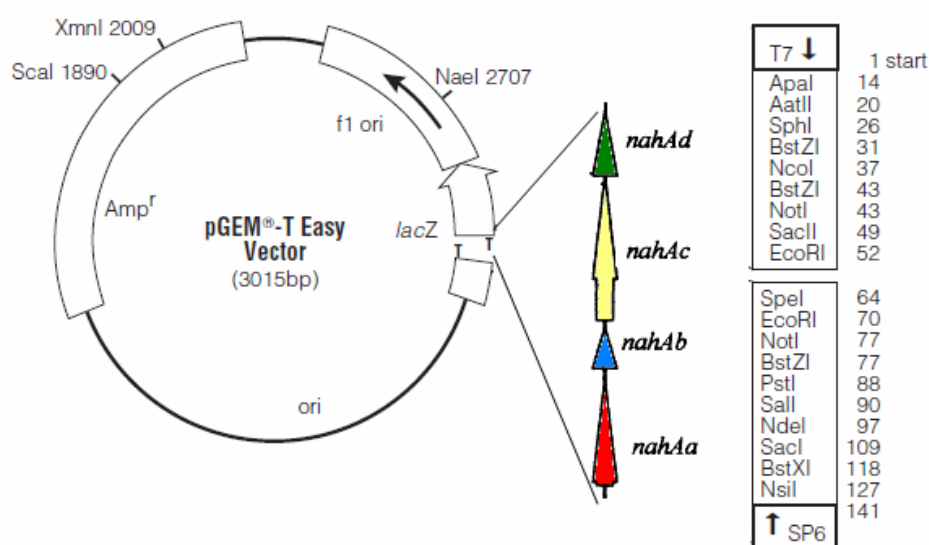


Fig. 4.2. Cloning map of NDO cluster from N1 and N2 strains. (pCZ2 and pCZ3 plasmids)

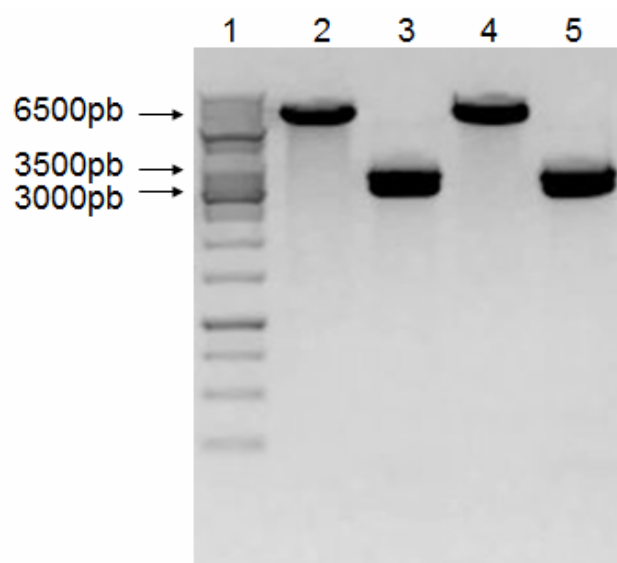


Fig. 4.3. pCZ2 and pCZ3 restriction analysis. Lane 2: pCZ2 NdeI digestion; lane 3: pCZ2 XhoI-SacI digestion; lane4 pCZ3 NdeI digestion; lane 5 pCZ3 Xho-SacI digestion.

Table 4.5. Analysis of the sequences cloned in pCZ2 and pCZ3.

ORFs	nucleotide	genes	aminoacid number	aminoacidic similarity (%)		subunits
				<i>P. aeruginosa</i>	<i>P. stutzeri</i>	
orf 1	1-986	NahAa	329 aa	100%	99%	Fe-S flavoreductase
orf 2	1131-1446	NahAb	105 aa	100%	100%	2Fe-2S ferredoxin
orf 3	1515-2864	NahAc	450 aa	99%	97%	NDO-H α subunit
orf 4	2879-3460	NahAd	194 aa	100%	99%	NDO-H β subunit

4.1.2. Cloning of phenanthrene dioxygenase (PhDO) genes from *Sphingobium* sp.

4.1.2.1. *Sphingobium* sp. PhS characterisation

Sphingobium sp. PhS, previously isolated from a polluted soil for its ability to grow using PAHs as the sole carbon and energy source, was identified by 16S rDNA analysis [97]. Its dose-dependent growth behaviour on phenanthrene in a biphasic system was evaluated: the substrate concentration of 1 mM allowed the highest increase in cell concentration after 3 days of incubation. Substrate range showed good catabolic versatility of *Sphingobium* sp. PhS: phenanthrene and naphthalene allowed a more evident biomass increase than toluene, anthracene and fluorene. To evaluate the strain's ability to produce biofilm, a number of assays were performed. Growth in an adherent form was found to be dose-dependent: phenanthrene 4 mM determined a higher yield in biofilm formation after 5 days of static incubation (BI = 2) than both phenanthrene 2 mM (BI = 1) and phenanthrene 1 mM (BI = 0.9). A chemotactic behaviour enhances the ability of motile bacteria to locate and degrade low concentrations of toxic, although metabolisable, compounds present in contaminated environments. A preliminary soft agar plate assay showed a chemotactic response of *Sphingobium* sp. PhS to phenanthrene [91].

These results suggested that *Sphingobium* sp. PhS was able to carry encoding genes for a peripheral oxygenase involved in phenanthrene degradation; therefore, it was decided to verify the presence of PhDO in this strain.

4.1.2.2. PhDO: gene cloning and preliminary functional characterisation

Unlike other oxygenases, PhDO genes are not clustered: in the bacterial genome catalytic-subunit encoding genes are usually located in different positions to electron-transporter encoding genes [49]. The procedure to clone oxygenase encoding genes from the previously described strains was carried out through the amplification and sequencing of a small conserved region of the large subunit of the hydroxylating subcomplex, the design of new primers based on the sequences of the oxygenase showing the highest degree of similarity and the cloning of the amplification product. The approx. 300 bp fragment, amplified with the primers Phn321F and Phn 617R, displayed a high degree of similarity with the *bphA1f* gene of *Novosphingobium aromaticivorans*. On these bases, it was assumed that all genes of the *bph* series would show similar similarity. Therefore, primers

were designed on *bphA1fA2f* (hydroxylating subcomplex f), *bphA1aA2a* (hydroxylating subcomplex a), *bphA3* (ferredoxin) and *bphA4* (ferredoxin reductase) published sequences of *N. aromaticivorans*. The amplified fragments showed a high similarity (95 to 98%) with the analogous *N. aromaticivorans* genes. The amplicons were cloned, step by step, into pGEM series vectors in order to obtain pSG6 (*bphA3A4A1fA2f*) and pRO2 (*bphA3A4A1aA2a*) plasmids, each carrying genes encoding for the entire enzymatic complex. For better protein expression the two fragments were then excised and transferred to pET series vectors, thus, pRO3 (*bphA3A4A1fA2f*) and pRO4 (*bphA3A4A1aA2a*) plasmids were obtained. pRO3 and pRO4 were cloned in a *E. coli* ER2566 heterologous host and used to carry out expression and preliminary biotransformation experiments. However, neither the gene product analyses (SDS-PAGE) nor the preliminary phenanthrene biotransformation assays gave satisfactory results. A detailed analysis of the sequences cloned in these two plasmids did not revealed any error that could have compromised their expression. To verify the functionality of the subunits, and to clarify the above results, new plasmids were constructed: *bphA1aA2a* was excised from the pRO4 plasmid and transferred to the pET vector to obtain the new plasmid pNC4. In addition, the pEM plasmid series was constructed [95]. The expression products of the pNC4 and pEM series plasmids were evaluated by SDS-PAGE: results showed that both the catalytic subunits and the *bphA3* electron transporter were correctly expressed (data not shown), although the *bphA4* expression was not observed. However, pNC4 and pEM series plasmids were used to create two hybrid expression systems (PhDO/NDO). Preliminary biotransformation assays were performed on naphthalene and phenanthrene using *E. coli* ER2566 as the heterologous host; each *E. coli* strain carried two plasmids in different configurations as shown in Table 4.6.

The biotransformation showed that none of the expression systems containing genes encoding for the PhDO catalytic subunit led to a functional enzyme. On the bases of these results it was decided not to carry out further biotransformations assays with this enzyme.

Table 4.6. Biotransformation assays performed using *E. coli* ER2566

cotransformation	features	vectors	reconstituted ioxygenase	indigo production	biotransformation	
					substrate	result
pEM3 +	<i>bphA3A4</i> +	pSTV28 +	bpha	-	biphenyl	-
pNC4	<i>bphA1aA2a</i>	pET-30			phenanthrene	-
pEM 13 +	<i>bphA3+RBS+bphA4</i> +	pET-30 +	bpha	-	biphenyl	-
pEM10	<i>bphA1aA2a</i>	pSTV28			phenanthrene	-
pEM13 +	<i>bphA3+RBS+bphA4</i> +	pET-30 +	bpha	-	biphenyl	-
pEM9	RBS + <i>bphA1aA2a</i>	pSTV28			phenanthrene	-
pEM7 +	<i>nahAaAb</i> +	pET30 +	NDO	++	naphthalene	+
pEM14	<i>nahAcAd</i>	pSTV28				
pEM14	<i>nahAcAd</i>	pSTV28	NDO hydroxylase	+	naphthalene	+
pEM5 +	<i>nahAaAb</i> +	pSTV28 +	hybrid enzyme	-	biphenyl	-
pNC4	<i>bphA1aA2a</i>	pET30			phenanthrene	-
pEM7 +	<i>nahAaAb</i> +	pET-30 +	hybrid enzyme	-	phenanthrene	-
pEM9	RBS + <i>bphA1aA2a</i>	pSTV28				
pEM13 +	<i>bphA3+RBS+bphA4</i> +	pET-30 +	hybrid enzyme	+	naphthalene	+
pEM14	<i>nahAcAd</i>	pSTV28				

4.2. Design and construction of toluene/*o*-xylene monooxygenase mutants

In a previous work [101] it was discovered that a TouA mutant, E214G/D312N/M399V isolated by the saturation mutagenesis, was able to oxidize nitroaromatic substrates with a higher efficiency than that of wild-type ToMO. TouA positions D312 and M399 did not appear to play an important role in catalysis whereas E214G was responsible for the enhanced rate of nitroaromatic oxidation. TouA variants E214G, E214A and E214V oxidized *o*-nitrophenol and formed 3-nitrocatechol 2.1, 1.6, and 1.2 times faster than the wild-type ToMO, respectively, whereas variant E214W formed 3-nitrocatechol 3 times slower. The mutants with R groups roughly equivalent in size to glutamate, E214Q and E214F, oxidized *o*-nitrophenol at the same rate. These results suggested that position E214 is a gate amino acid controlling the rate of nitroaromatic oxidation. The 3D structure analysis, carried out by this laboratory, confirmed the hypothesis that E214, situated more than 10 Å away from the diiron centre, is potentially involved in regulating the flow of molecules into the active site. In particular, two residues located at the channel entrance, E214 and D211, seemed to be able to block the transit of molecules toward the reaction centre through hindrance (Fig. 4.4 a). The residue in position 211 is located in an alpha helix; aspartate was chosen to replace alanine because of its low hindrance characteristics and high alpha propensity.

In-silico 3D analysis of D211A and E214G/D211A substitutions confirmed the preliminary observations (Fig. 4.4 b). E214G/D211A molecular surface representation showed the wide cavity caused by the fusion of the two openings. By a preliminary *in-silico* docking approach, performed using Monte Carlo – Simulated Annealing algorithm, the ability of many aromatic compounds, including molecules larger than toluene, to pass through the opening created by the above-mentioned mutations was tested. The free energy docking values obtained by simulations on nitrobenzene, quinoline, anisole, aniline, biphenile, benzophenone, trans-stilbene, and 1,2,3-trimethoxybenzene, were compatible with transient interactions across the opening in which the molecules were able to fit. On the bases of these results it was hypothesised that the mutants should allow better transit of certain substrates and thus they are able to display a spectrum of substrates different than that of the wild-type (Fig. 4.5). The D211A and D211A/E214G mutants were constructed

by site directed mutagenesis and were found to be functional in respect of oxidation of indole to indigo. DNA sequencing confirmed the presence of the desired mutations [72].

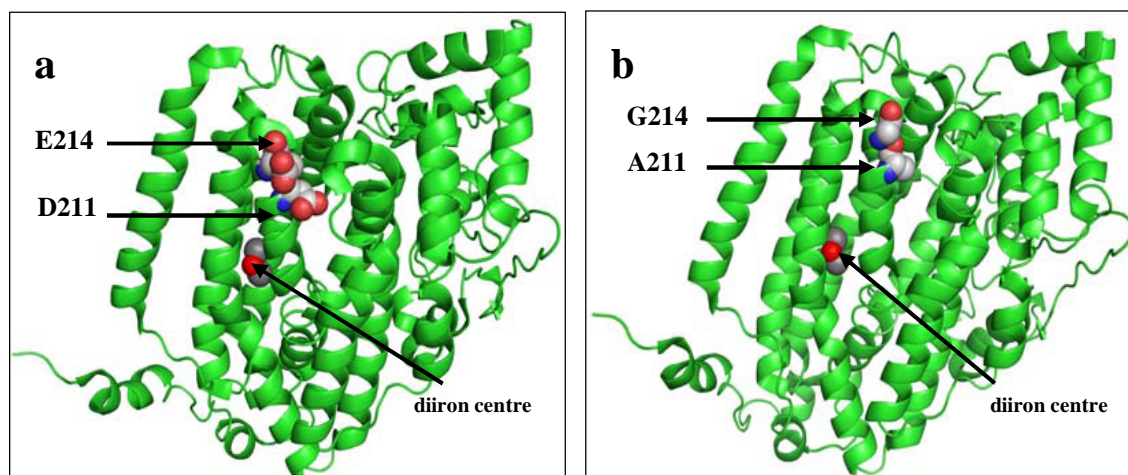


Fig. 4.4. Ribbon representation of the TouA subunit. The diiron centre is located in a wide hydrophobic pocket. At the channel opening level, D211 and E214 residues (wild-type) (a) show more hindrance than that the A211 and G214 residues (b).

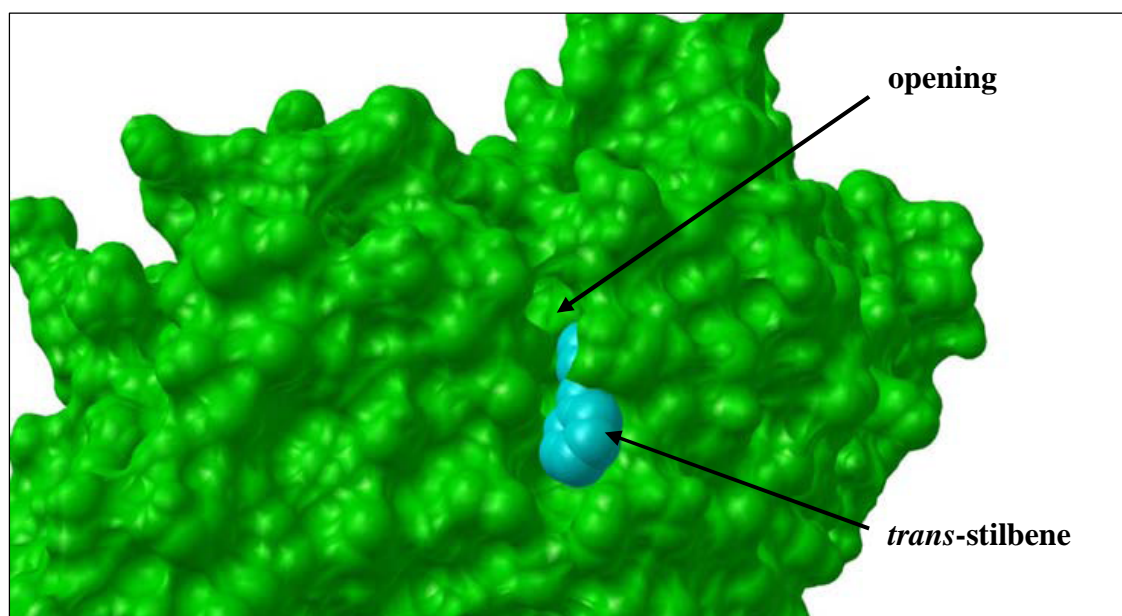


Fig. 4.5. Docking simulation performed using Monte Carlo - Simulated Annealing algorithm on D211A/E214G mutant using *trans*-stilbene.

4.3. Biotransformation assays

4.3.1. Biotransformation protocol optimisation

The biotransformation protocol represents a key step towards obtaining a good reaction yield. In this study, two main protocol steps were optimised: cell growth/induction, and the substrate addition method.

4.3.1.1. Cellular growth and induction

To optimize cellular growth, either LB medium or M9 minimum medium were used, depending on the bacterial strain.

The LB medium led to a rapid and consistent bacterial growth but was not the optimal choice when further analyses were performed by GC-MS or NMR because the presence of many different substances in the medium can affect results by causing high background noise. The LB medium was, therefore, only used for strains that showed poor growth in M9 minimum medium, such as *E. coli* carrying plasmids encoding for both ToMO and ToMO variants. To obtain an O.D._{600nm} 0.1 starting culture, the LB medium was inoculated with the culture cells grown o.n.. After 2 hours of incubation, at O.D.₆₀₀ of 0.6, the culture was induced with IPTG 1mM and culture samples were collected after 30, 60, 90, 120, 150, 180, 210 and 240 minutes of growth. The indigo assay showed that the optimal induction time was 120 minutes: a colorimetric test performed to evaluate the indigo production showed that at this time the highest amount of indigo precipitate was observed. Compared to the LB medium, the M9 minimum medium guarantees a growth environment devoid of substances that can potentially affect GC-MS analyses. This protocol was, therefore, applied for strains that showed good growth in a mineral medium in the presence of a single hydrocarbon as the sole source of carbon and energy, such as *E. coli* JM109 carrying the NDO encoding plasmid. Here also, the best optimal induction time was 120 minutes.

The induced cells were collected and stored for the further biotransformation experiments. For each biotransformation, reaction mixture was prepared using a flask in which the induced cells were resuspended in M9 mineral medium containing glucose at the concentration of 1mM (to promote the reducing power production). The biotransformation reaction starts when the substrate is added to the cellular suspension (t0).

4.3.1.2. Substrates addition

The yield of a biotransformation containing cells in the water phase is strongly influenced by physical and chemical substrate features. For each compound a specific optimisation of the whole protocol should be used before performing biotransformation assays. In this analysis three compounds were used as pilot molecules, and each one represented a particular physic-chemical category.

To standardise the substrate addition methods, only the wild-type ToMO expressed in the *E.coli* JM109 heterologous host was used. Using the growth and induction conditions previously described, a number of ToMO biotransformation assays were performed. Toluene and nitrobenzene were used as pilot substrates to standardise the “water insoluble-liquid-volatile” and “water soluble-liquid” substrate addition methods, respectively. Biphenyl was used to standardise the “water insoluble-solid” substrate addition method.

Standardisation of the water insoluble- liquid- volatile substrate addition method

Toluene, the ToMO natural substrate, is a lipophilic and volatile compound. It is able to create an organic phase on the medium surface and subsequently evaporate, so was, therefore, added to the medium as a vapour using a special flask in order to provide a saturating condition. Preliminary biotransformation results showed that this protocol gives a satisfactory biotransformation yield (Fig. 4.6). The biotransformation product, a cresols mixture, was identified and quantified by HPLC analyses. The toxicity test, performed by plating t0' and t1440' collected samples, showed that the chosen conditions were not toxic (t0', $5 \cdot 10^8$ CFU/ml – 1440', $4 \cdot 10^8$ CFU/ml)

An alternative method based on a biphasic system, by means of an isooctane (i.o.) organic phase, to which toluene (100 mM) was added, was also used. Results showed that the ToMO specific activity was lower than that observed when using the first approach (Fig. 4.6), therefore, the former methodology was applied for the subsequent toluene biotransformations.

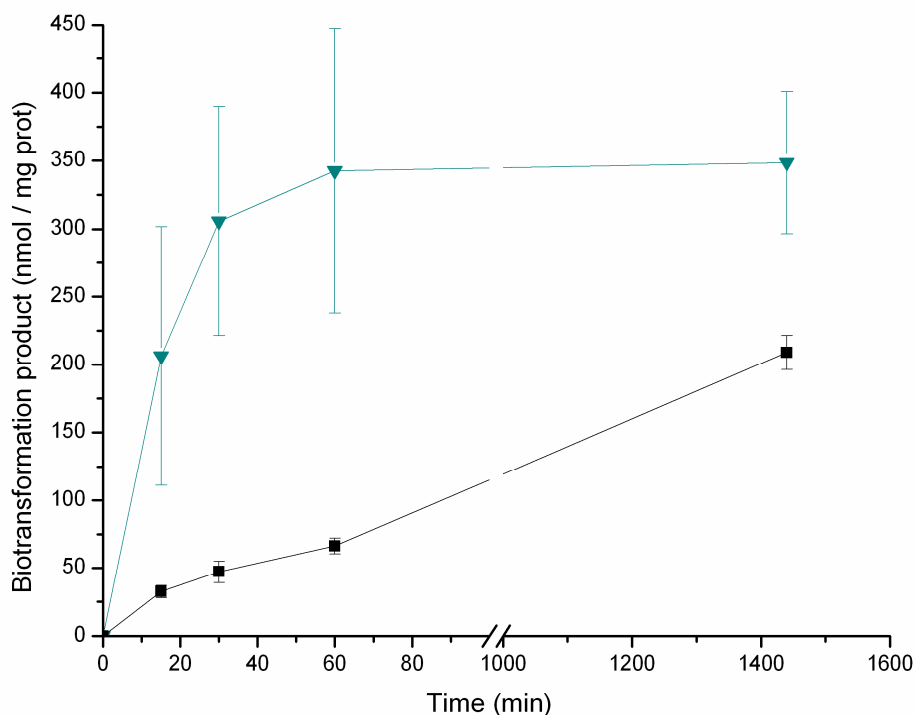


Fig. 4.6. Biotransformation of toluene by ToMO. (▼) Substrate added as a vapour; (■) biotransformation in biphasic system. Data are expressed as nmol of product / mg of protein.

Standardisation of water soluble- liquid substrate addition method

Nitrobenzene, an aromatic soluble substrate, was added to the medium as a pure chemical at a concentration of 0.2 mM. A HPLC signal, compatible with a product more oxidised than the substrate, was observed although its retention time did not correspond to any nitrophenol isomer. However, quantitative results calculated on the nitrobenzene disappearance showed that the biotransformation yield was satisfactory: a nitrobenzene decrease of approximately 65% of that present at t_0 was observed. The toxicity test, performed by plating t_0' and t_{1440}' wild-type collected samples, did not show any toxic effect (t_0' , $3,5 \cdot 10^8$ CFU/ml – t_{1440}' , $2,6 \cdot 10^8$ CFU/ml). On the basis of these results, this substrate addition method was chosen for all the soluble aromatic hydrocarbons used in this study.

Standardisation of water insoluble- solid substrate addition method

Biphenyl is an insoluble compound carrying two aromatic rings. In order to standardise the substrate addition method, three biphenyl biotransformation assays were carried out:

Biphasic system

The first assay was performed using a biphasic system in which the water phase and organic phase ratio was 7:1. A biphenyl solution at a concentration of 100 mM was prepared using i.o. as an organic solvent. HPLC analysis results, expressed in absorbance units normalised on total proteins, are reported in Figure 4.7. The toxicity test, performed by plating t0' and t1440' wild-type collected samples, showed that the chosen conditions were not lethal (t0', $3.1 \cdot 10^8$ CFU/ml – t1440', $2.8 \cdot 10^8$ CFU/ml).

DMSO-based protocol

Dimethyl sulfoxide (DMSO) is a compound capable of making biological membranes permeable; this particular characteristic was used to develop a more efficient biotransformation protocol.

Two different assays were carried out:

1. in the first experiment the induced cells were resuspended in phosphate buffer (PB)/DMSO with a 9:1 ratio and then incubated for 10 minutes. The cells were then washed in PB and the previously described 7:3 biphasic system based protocol was applied.
2. in the second experiment, DMSO was directly added at the water phase before adding the substrate: in order to obtain a 7:3 water phase /organic phase ratio, 1 ml of DMSO and 6,8 ml of biphenyl solution in isooctane (100 mM) were added to 15,9 ml of cellular suspension.

The incubation conditions were the same as in the previous experiments. In both assays, to preserve the water phase/organic phase ratio, the samples were collected as follows: t0 (2 ml of water phase; t60 (2 ml of water phase and 0,8 ml of organic phase); t1440 (2 ml of water phase and 0,9 ml of organic phase). Results are reported in Figure 4.7. In both assays no toxic effect was observed (1st experiment: t0', $2.4 \cdot 10^8$ CFU/ml – t1440', $1.3 \cdot 10^8$ CFU/ml; 2nd experiment: t0', $4.2 \cdot 10^8$ CFU/ml – t1440', $3.8 \cdot 10^8$ CFU/ml).

Although DMSO is a water soluble molecule its methyl groups can facilitate the solubilisation of water-insoluble organic compounds. On this basis, a new biotransformation assay was prepared: 1 ml of 20 mM biphenyl – DMSO solution was

added to 19 ml of a.ph. cellular suspension. The DMSO final concentration (not enough for a complete biphenyl water solubilisation) was calculated on the basis of DMSO toxicity assay results. The reaction was incubated as described in the previous assays and 1 mL of the water phase. sample was collected at the times: t0; t60; t240; t1440 minutes.

HPLC analysis results showed that this method had a yield comparable to other DMSO-based tested protocols (Fig. 4.7).

Addition of pure chemical biphenyl

The final tested biphenyl biotransformation assay was carried out by adding the pure substrate directly to the water phase. cellular suspension: 30 mg of biphenyl in 20 ml of water phase. cellular suspension. The reaction was incubated as in the previous assays and 1 ml of sample was collected at the times: t0; t60; t240; t1440 minutes. HPLC analyses showed that this biphenyl biotransformation protocol allowed a higher product yield compared to that previously tested (Fig. 4.7). Additionally, under these operative conditions, no toxic effect was observed (t0', $3,0 \cdot 10^8$ CFU/ml – t1440', $2,8 \cdot 10^8$ CFU/ml). This substrate addition method was therefore chosen for all the insoluble-solid aromatic hydrocarbons used in this study.

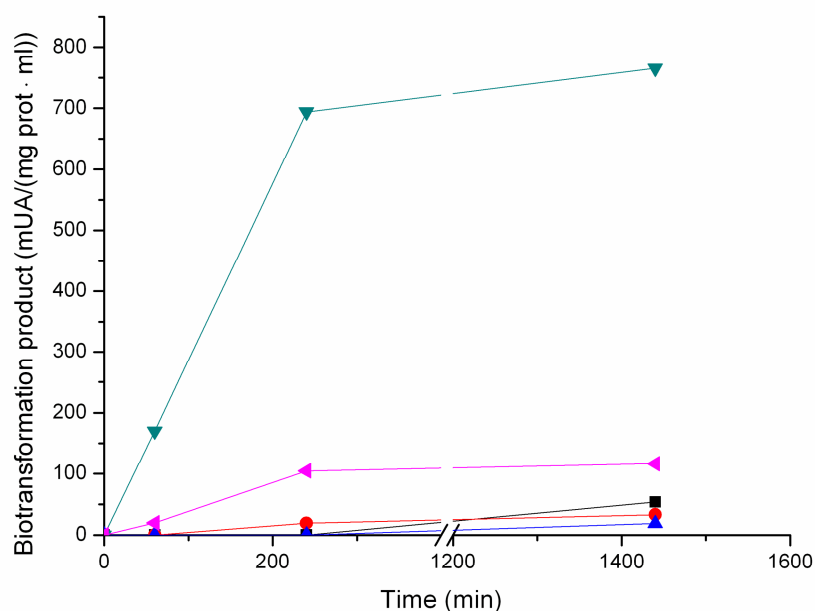


Fig 4.7. Biotransformation of biphenyl by *ToMO*. The substrate was added: using a biphasic system (●); using a biphasic system after treatment of the cells with TP/DMSO (▲); using a biphasic system in which the water phase was supplied with DMSO (■); to the water phase in the presence of DMSO (◄); to the water phase as a pure chemical (▼). Data are expressed as mU of Absorbance / (mg of total protein · ml).

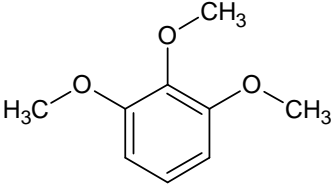
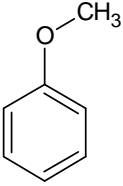
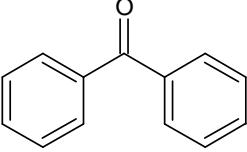
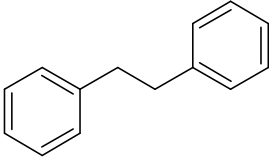
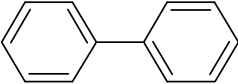
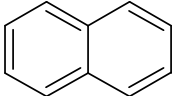
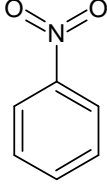
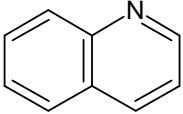
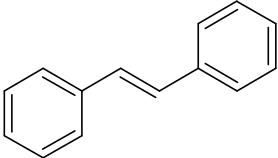
4.3.2. Biotransformation product analyses

The ability of *T_oMO* and NDO to hydroxylise anisole, benzophenone, bibenzyl, biphenyl, nitrobenzene, quinoline, trans-stilbene and 1,2,3- trimethoxybenzene (Table 4.7) was tested by biotransformation assay. These substrates were chosen for their environmental, pharmacological and biotechnological importance and to characterise the substrate range of NDO and *T_oMO*, respectively.. In addition, the ability of *T_oMO* mutants to hydroxylise the same substrates was evaluated; E214G was used as a reference mutant [71]. A naphthalene biotransformation assay was performed for NDO only.

T_oMO and NDO biotransformation products were characterised by GC-MS analyses. This technique requires the product extraction from water phase. samples by an organic solvent; ethyl acetate (EtAc) was used to obtain an 1:1 water phase/EtAc ratio. Three extraction methods were tested: 1) from the water phase. after centrifugation; 2) from the water phase. with the cells present in suspension; 3) after lyophilisation of the sample. Preliminary GC-MS analysis, aimed at verifying the efficiency of the above three extraction methods, suggested that the most efficient method was the second tested (with the cells present in suspension), which was therefore used for all further biotransformation assays. The GC-MS analyses were performed using the FullScan method, the most suitable instrument preset to work with unknown compound mixtures.

Both *T_oMO* and NDO biotransformation products were quantified by HPLC. This technique was chosen because, for precise GC-MS quantification, we needed to use internal deuterated standards that were not available in the laboratory when the analysis was carried out. With the exception of toluene, a proper kinetic analysis was not carried out for the substrates tested, instead, a quantitative evaluation of the products after a reaction time of 24 hours was performed.

Table 4.7. Substrates used in NDO and T₀MO biotransformation assays.

Substrate	GC-MS retention time (min)	Structure
1,2,3-trimethoxybenzene	5,97	
Anisole	3,80	
Benzophenone	7,45	
Bibenzyl	6,99	
Biphenyl	7,75	
Naphthalene	5,44	
Nitrobenzene	4,88	
Quinoline	5,70	
<i>trans</i> -stilbene	7,84	

4.3.2.1. Characterisation of ToMO and TouA mutants' biotransformation products

Biotransformation assays using ToMO and TouA mutants were carried out: the LB medium-based protocol and, for each substrate, the chosen addition method were used. Only two samples were collected for each assay: t0 and t1440 (24 hours) minutes. The ToMO and TouA mutants' biotransformation products were characterized by GC-MS analyses. (Table 4.8). For each sample, after a retention time (r.t.) of 2 minutes, a strong signal, ascribable to cellular metabolites, was observed. However, using GC-MS software it was possible to remove undesired signals and background noise; this approach was used to edit chromatograms in all the biotransformations. For each substrate tested, the approximate relative abundance of the isomer produced was indicated on the basis of the observed chromatogram areas.

ToMO biotransformations

ToMO was able to hydroxylise all the substrates tested. From 1,2,3-trimethoxybenzene, as from anisole, all possible isomers were obtained (Fig. 4.8 – App. 4.4.a). In the 1,2,3-trimethoxybenzene biotransformation, it appeared that 2,3,4-trimethoxyphenol was more abundant compared to 3,4,5-trimethoxyphenol. In the anisole (App. 4.4.a), bibenzyl and biphenyl biotransformations the *para* isomer was the most abundant. Preferentially, benzophenone, nitrobenzene and *trans*-stilbene (App. 4.4.b) biotransformations caused the *meta* isomer formation; furthermore, the *ortho* isomer was never observed. Surprisingly, the quinoline biotransformation product was N-oxoquinoline, derived from the oxidation of the heteroatom (Fig. 4.9).

TouA mutant biotransformations

The TouA mutants' qualitative biotransformation results showed that only the E214G mutant was able to hydroxylate all the tested substrates. This mutant showed the same regio-preferences as the wild-type with the only difference observed being in the 1,2,3-trimethoxybenzene biotransformation where the most abundant isomer produced by E214G was 3,4,5-trimethoxyphenol and the most abundant isomer produced by wild-type was 2,3,4-trimethoxyphenol (Fig. 4.10).

D211A and E214G/D211A mutants were able to hydroxylate only some of the tested substrates although with a lower efficiency than the wild-type. No product was observed in either the D211A, D211A/E214G *trans*-stilbene, nitrobenzene or quinoline biotransformations. Bibenzyl was transformed by D211A/E214G but not by D211A.

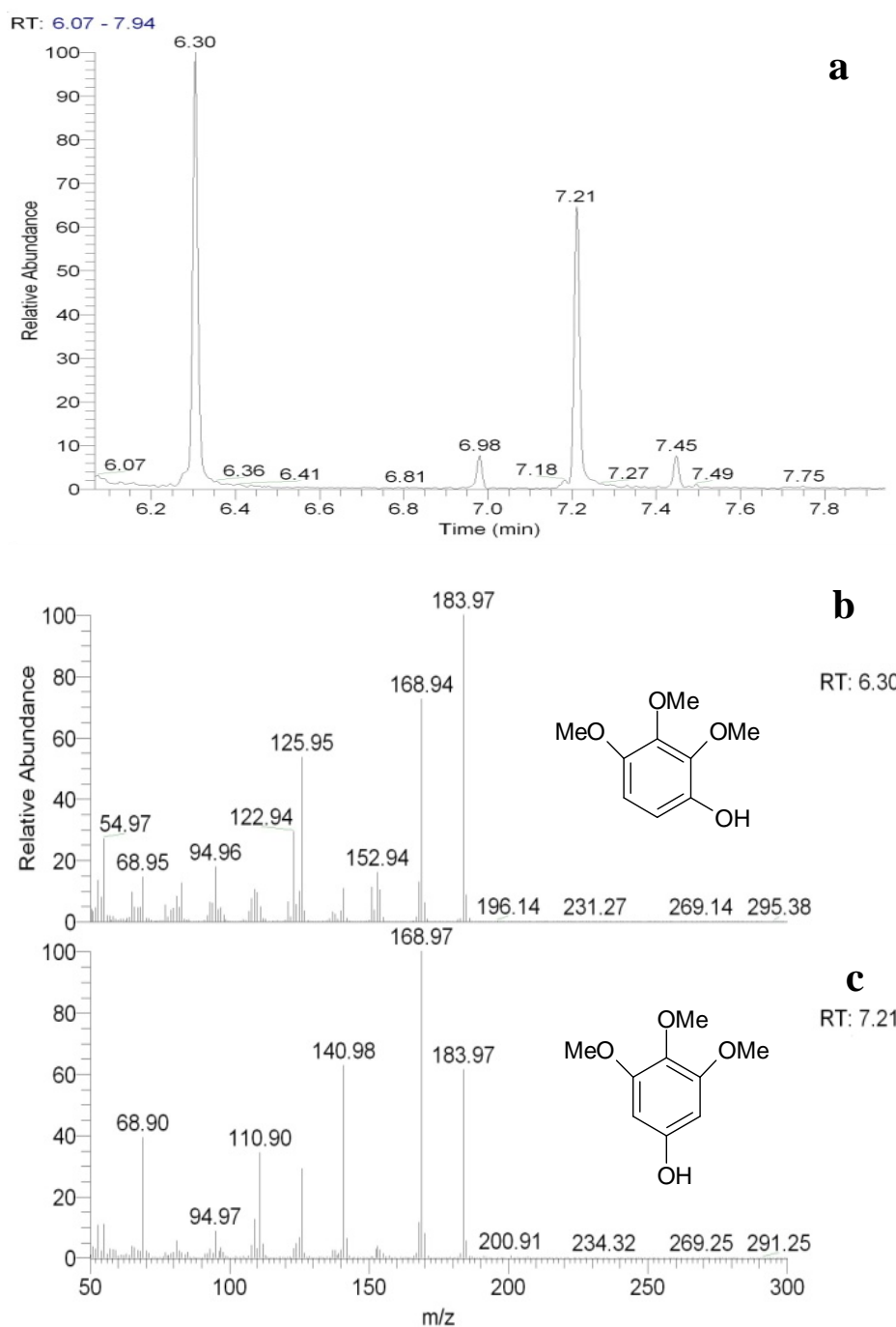
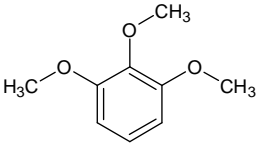
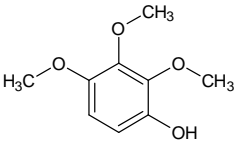
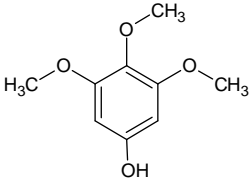
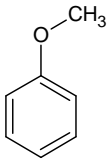
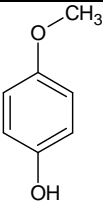
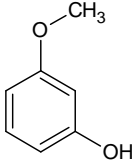
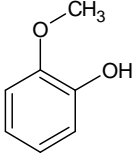
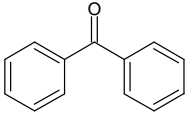
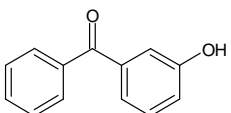
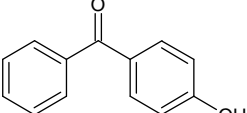
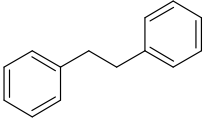
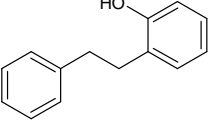
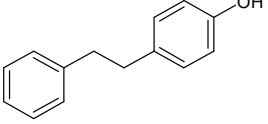
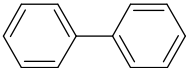
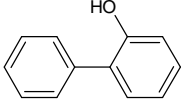
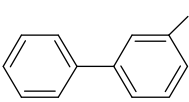
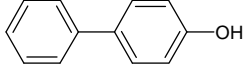
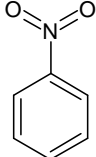
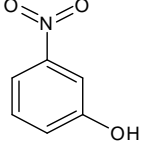
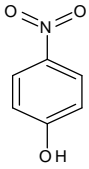
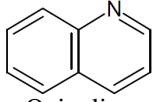
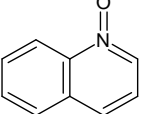
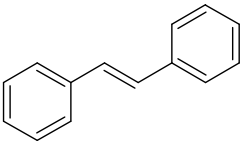
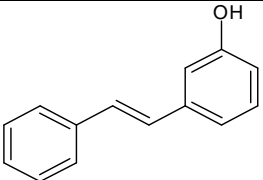
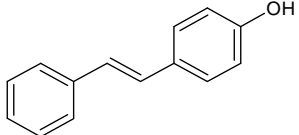


Fig. 4.8. GC-MS analysis of 1,2,3-trimethoxybenzene biotransformation product by *ToMO*. **a**) Chromatogram; **b**) 2,3,4-trimethoxyphenol (r.t. 6.30') and **c**) 3,4,5-trimethoxyphenol (r.t. 7.21') mass spectrum.

Table 4.8. ToMO biotransformation products.

Substrate	Products		
 1,2,3-trimethoxybenzene	 2,3,4-trimethoxyphenol	 3,4,5-trimethoxyphenol	
 Anisole	 4-methoxyphenol	 3-methoxyphenol	 2-methoxyphenol
 Benzophenone	 3-hydroxybenzophenone	 4-hydroxybenzophenone	
 Bibenzyl	 2-hydroxybibenzyl	 4-hydroxybibenzyl	
 Biphenyl	 2-hydroxybiphenyl	 3-hydroxybiphenyl	 4-hydroxybiphenyl
 Nitrobenzene	 <i>m</i> -nitrophenol	 <i>p</i> -nitrophenol	
 Quinoline	 N-oxoquinoline		
 <i>Trans</i> -stilbene	 3-hydroxy- <i>trans</i> -stilbene	 4-hydroxy- <i>trans</i> -stilbene	

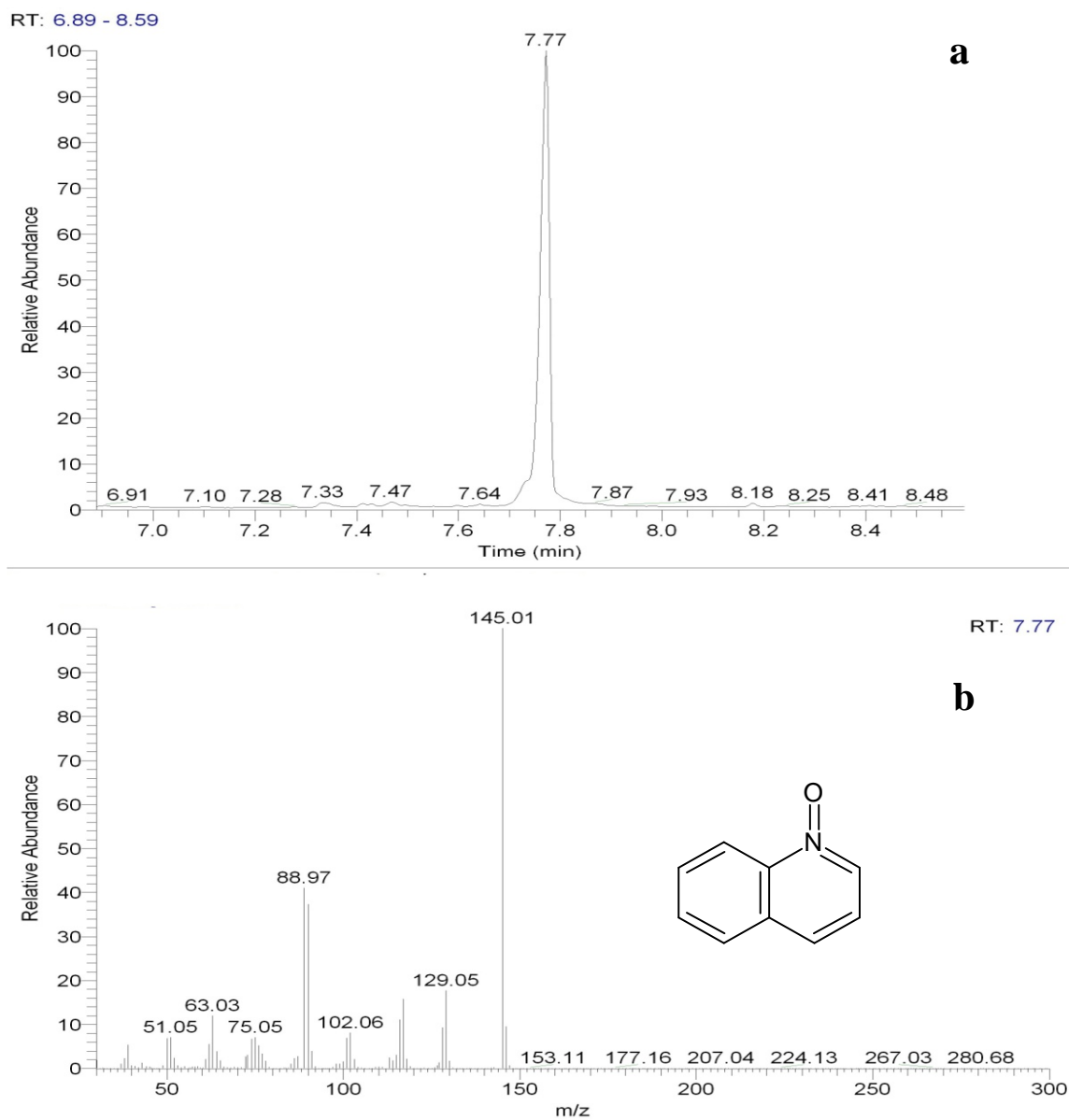


Fig. 4.9. GC-MS analysis of quinoline biotransformation product by *T_oMO*. **a)** chromatogram; **b)** N-oxoquinoline (r.t. 7.77') mass spectrum.

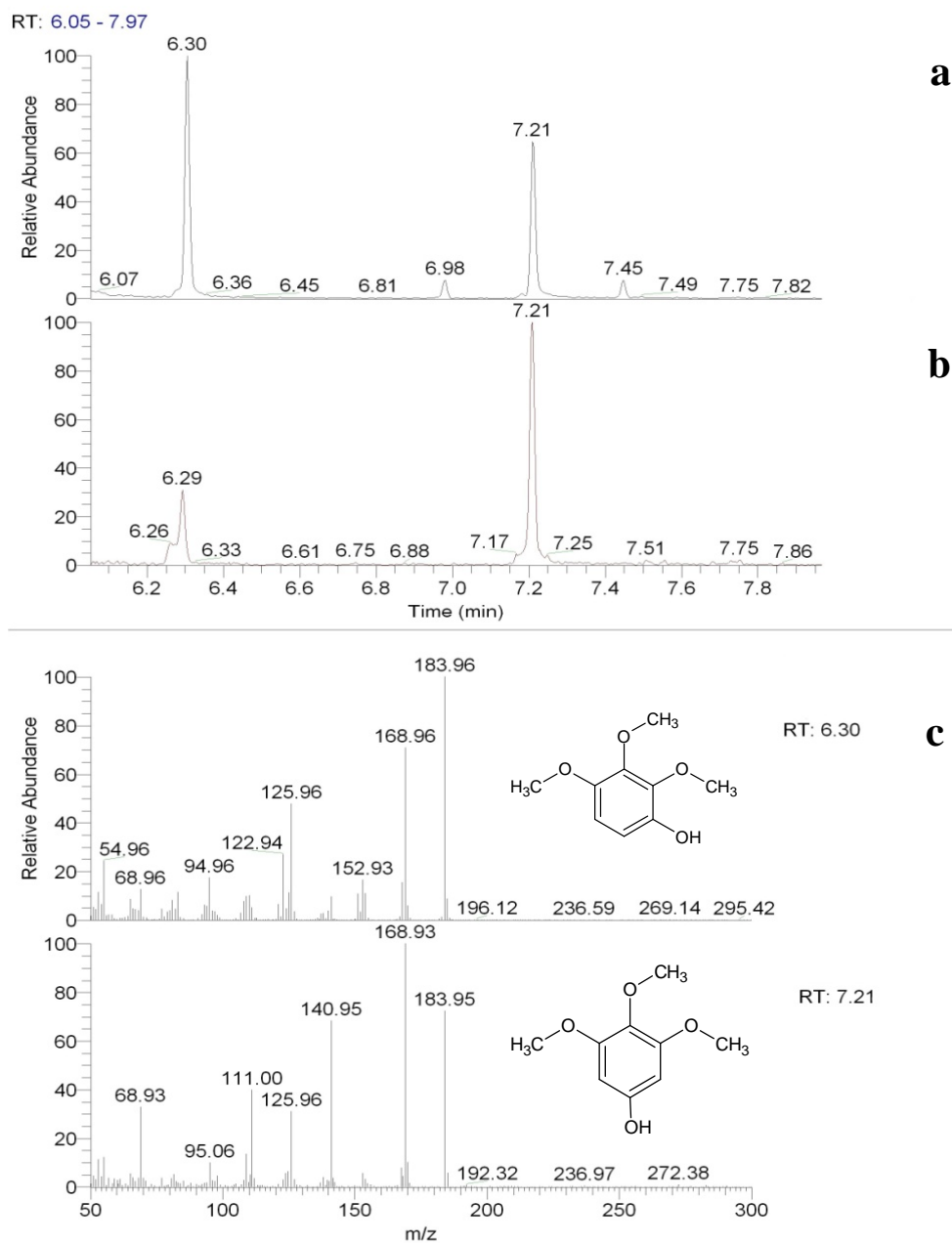


Fig. 4.10. GC-MS analysis of 1,2,3-trimethoxybenzene biotransformation product by E214G mutant. **a)** 1,2,3-trimethoxybenzene chromatogram by wild-type; **b)** 1,2,3-trimethoxybenzene chromatogram by E214G; **c)** 2,3,4-trimethoxyphenol (r.t. 6.30') and 3,4,5-trimethoxyphenol (r.t. 7.21') mass spectrum.

4.3.2.2. Quantification of *ToMO* and *TouA* mutants' biotransformation products

The activity of all *ToMO* variants on toluene, which is its natural substrate was evaluated; a kinetic analysis was carried out using the samples collected at the times t_0 , t_{15} , t_{30} , t_{60} and t_{1440} minutes. HPLC analyses showed that wild-type and E214G had a similar specific activity, 6.9 and 5.0 nmol/(min · mg of total protein) respectively; D211A and D211A/E214G showed a specific activity of 1.5 and 1.2 nmol/(min · mg of total proteins) respectively, both lower compared to that of the wild-type (Fig. 4.11).

Quantitative analyses were performed in triplicate by HPLC for the new substrates: from each biotransformation assay, t_0 ' and t_{1440} ' samples were analysed. For each substrate, except for those already described in literature, the UV spectrum was fixed in order to find out the maximum absorption wavelength. The calibration curves were made using commercial standard; because the unavailability of the bibenzyl and trans-stilbene mono- and dihydroxylated standards, it was not possible to quantify the bibenzyl and trans-stilbene biotransformation products.

For each substrate, the isomer mixture was quantified by HPLC: Table 4.9 shows the estimated yield of *ToMO* and *touA* mutants at 24 hours. The yield of the nitrobenzene biotransformation was calculated for the wild-type and the mutant E214G only, which are the sole two variants capable of transforming this compound. Nitrophenols can also act as a substrate for *ToMO*, which actually compete with nitrobenzene after a reaction time of around 30'. Therefore, not having available the commercial chemical standard to carry out a quantification for times longer than 30', it was decided to measure the biotransformation yield at a reaction time of 30': the wild-type and the mutant E214G showed a yield of 34.8 ± 5.4 nmol/(mg of total protein) and 114.0 ± 9.0 nmol/(mg of total protein) respectively.

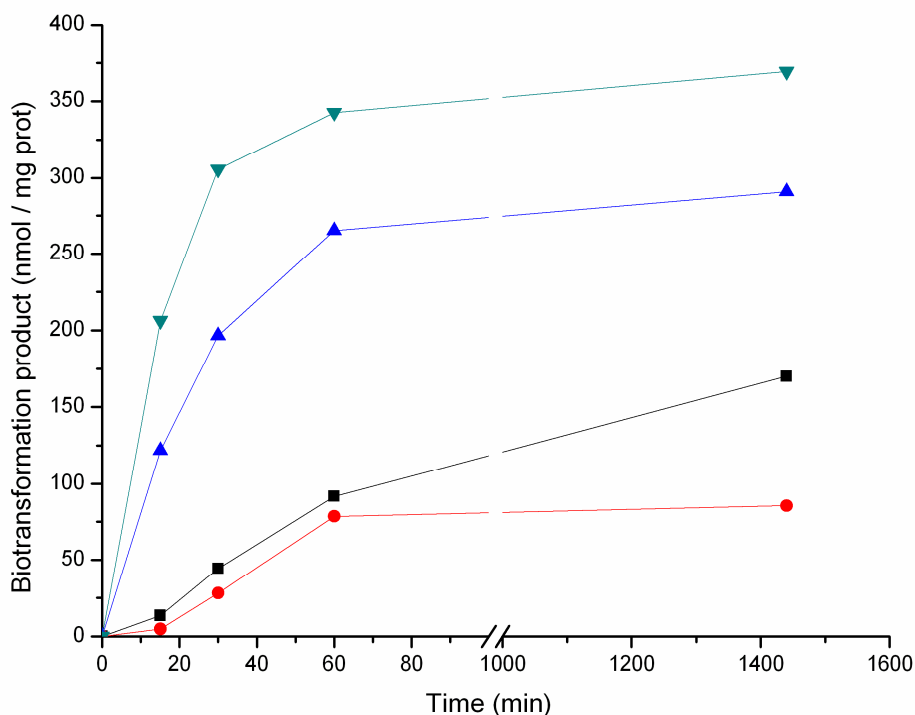


Fig. 4.11. Biotransformation of toluene by wild-type (▼), D211A (■), D211A/E214G (●) and E214G (▲). Data are expressed as nmol of product / mg of protein.

Table 4.9. ToMO variants yields at 24 hours determined by HPLC. Data are expressed in nmol/(mg of total protein).

Substrate/Product	Yield			
	wild-type	E214G	D211A	D211A/E214G
1,2,3-trimethoxybenzene/ trimethoxyphenol	89.9 ± 23.2	254.8 ± 25.3	19.2 ± 2.4	8.6 ± 1.8
anisole/ methoxyphenol	757.6 ± 288.5	1858.5 ± 903.6	103.3 ± 15.5	18.0 ± 6.8
benzophaenone/ hydroxybenzophaenone	30.7 ± 4.8	101.7 ± 3.8	44.5 ± 13.8	14.3 ± 1.6
biphenyl/ hydroxybiphenyl	82.6 ± 12.3	405.3 ± 45.8	33.8 ± 6.8	22.9 ± 3.1
quinoline/ N-oxoquinoline	954.0 ± 122.3	3097.5 ± 62.0	n.q.	n.q.

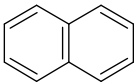
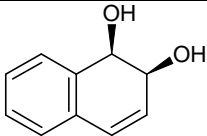
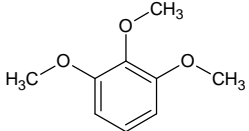
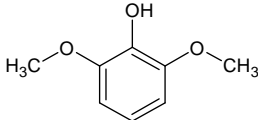
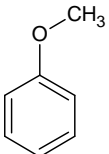
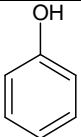
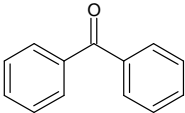
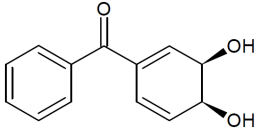
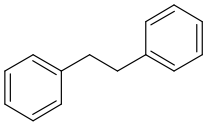
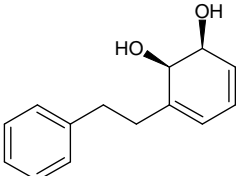
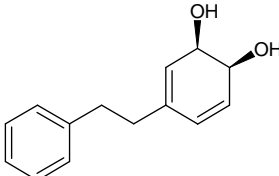
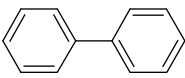
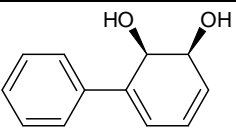
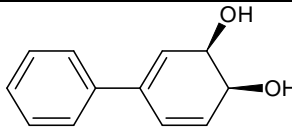
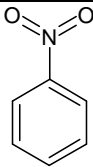
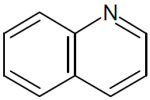
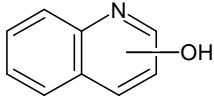
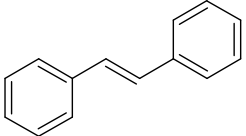
^a **Abbreviations:** n.q., not quantifiable.

4.3.2.3. Characterisation of NDO biotransformation products

Biotransformation assays were performed using the M9 minimum medium based protocols and, for each substrate, the chosen addition method. The *E. coli* JM109 strain carrying pCZ2 plasmid was used. Because of the likely different catalytic efficiency of NDO for each substrate tested, only two samples were collected for each biotransformation reaction: t0' and t1440'. GC-MS analyses showed that NDO was able to perform three different reactions: 1) dealkylation (anisole and 1,2,3-trimethoxybenzene), 2) monohydroxylation (quinoline), and 3) dihydroxylation (biphenyl, benzophenone and bibenzyl). Results of qualitative GC-MS analyses are showed in Table 4.10. The background noise, observed for each sample at r.t. 2', was removed by GC-MS software. Figure 4.12 shows the naphthalene biotransformation GC-MS analysis results: panels **a** and **b** display the uncut and the cleaned chromatogram respectively; panels **c** and **d** shown the mass spectrum of the identified biotransformation products 1-naphthol and 2 naphthol mixture (r.t. 6.99'), and 1,2-dihydroxy-1,2-dihydro-naphthalene (r.t. 7.13'). The presence of two naphthols is caused by the instability of the dihydro-diol that tends to dihydrate; in this way it restores the molecule aromaticity. Dehydration can happen both during the 24-hour incubation period and during the injection process due to the high temperatures of the GC-MS injector. The probability that an OH group in either position 1 or 2 would be lost during the reaction is equal. This explains why the analysis showed the presence of both isomers. Similarly, the benzophenone biotransformation product was 2,3-dihydro-2,3-dihydroxybenzophenone.

Each of the biphenyl and bibenzyl biostransformations produced different dihydroxylated isomers. Biphenyl biotransformation GC-MS results are shown in Figure 4.13: the signal at r.t. 7.93' (Panel **a**) represents the dihydroxylated product (Panel **b**); the signals at r.t. 7.77', 7.73' and 6.98' are ascribable to *orto*, *meta* and *para* monohydroxylated isomers respectively, derived, as for naphthalene biotransformation products, from a spontaneous dehydration reaction (App. 4.5.a). The probability of losing one of the OH groups from the dihydroxylated product is the same for each group, accordingly, the presence of the three monohydroxylated compounds indicates that the NDO biphenyl biotransformation products were 3-dihydro-1,3-dihydroxybiphenyl and 2,3-dihydro-2,3dihydroxybiphenyl mixture.

Table 4.10. NDO biotransformation products.

Substrate	Reaction	Products	
	Dihydroxylation	 1,2-dihydro-1,2-dihydroxynaphthalene	
	Dealkylation	 2,6-dimethoxyphenol	
	Dealkylation	 Phenol	
	Dihydroxylation	 2,3-dihydro-2,3-dihydroxybenzophenone	
	Dihydroxylation	 1,2-dihydro-1,2-dihydroxybibenzyl	 2,3-dihydro-2,3-dihydroxybibenzyl
	Dihydroxylation	 1,2-dihydro-1,2-dihydroxybiphenyl	 2,3-dihydro-2,3-dihydroxybiphenyl
	n.o.		
	Monohydroxylation	 hydroxyquinoline	
	n.o.		

^a Abbreviations: n.o., not observed.

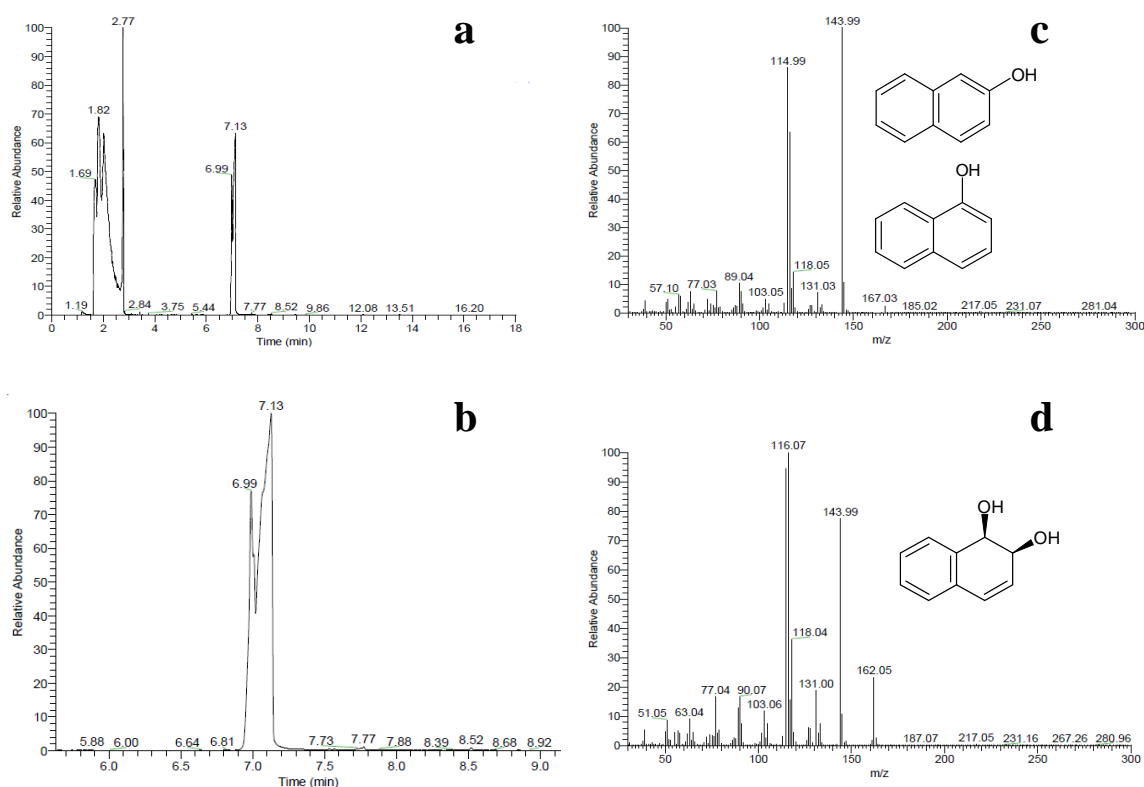


Fig. 4.12. GC-MS analysis of naphthalene biotransformation products by NDO. Panels **a)** and **b)** show the entire and the cut chromatogram respectively; **c)** 1-naphthol and 2 naphthol mixture mass spectrum; **d)** 1,2-dihydroxy-1,2-dihydronaphthalene mass spectrum.

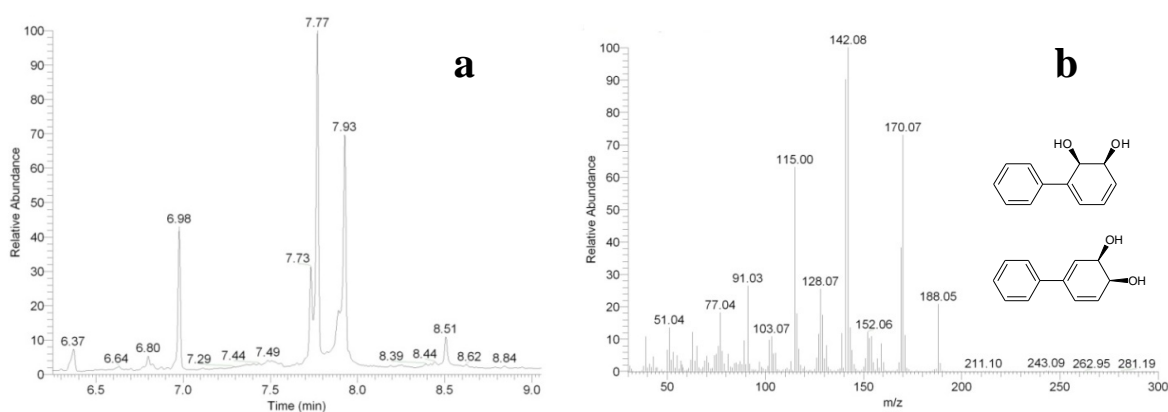


Fig. 4.13. GC-MS analysis of biphenyl biotransformation products by NDO. **a)** Chromatogram; **b)** mass spectrum of the signal at r.t. 7.93'.

On quinoline, a different reaction was observed: NDO products were a mixture of monohydroxylated isomers (App. 4.5.b).

On anisole and 1,2,3-trimethoxybenzene, a dealkylation reaction was observed. Anisole and 1,2,3-trimethoxybenzene biotransformation products were phenol and 2,3 / 2,6-dimethylbenzene mixture respectively.

No product was observed for the nitrobenzene and trans-stilbene biotransformations.

4.3.2.4. Quantification of NDO Biotransformation products

Quantitative analyses were performed in triplicate by HPLC: samples collected from each biotransformation assay at t0' and t1440' were analysed. The calibration curves were made using commercial standard; for the reasons described above (see S. 4.3.2.2), it was not possible to perform bibenzyl and trans-stilbene biotransformation product quantifications.

In biphenyl, benzophenone and naphthalene biotransformations only commercially unavailable isomers were obtained. However, after a mild treatment with H₂SO₄ and heating, these dihydroxylated compounds are able to recover aromaticity by dehydration (Fig. 4.14); this ability was used to perform an indirect quantification, which was carried out using the equivalent commercially available phenols. Fig. 4.15 shows the naphthalene biotransformation HPLC analysis; the chromatogram displayed a signal ascribable to dihydroxylated products at lower r.t. with respect to those of the monohydroxylated compounds. After the dehydration process, this signal decreased and, at the same time, the monohydroxylate compounds' signal increased, suggesting that the monohydroxylated signal additive value was dependent on the dehydration process of the dihydroxylated products.

The NDO yields at 24 hours calculated, for each substrate, on total products (isomer mixture), are shown in Table 4.11 .

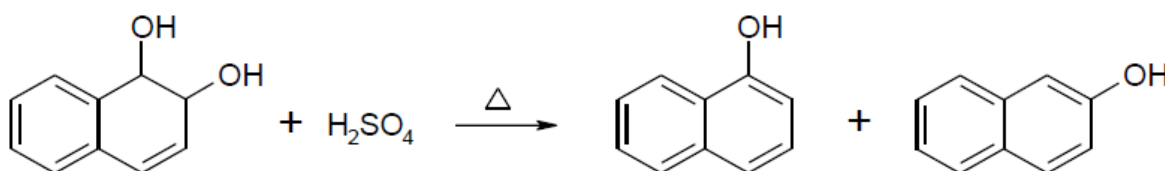


Fig. 4.14. Acid dehydration of 1,2-dihydro-1,2-dihydroxynaphthalene

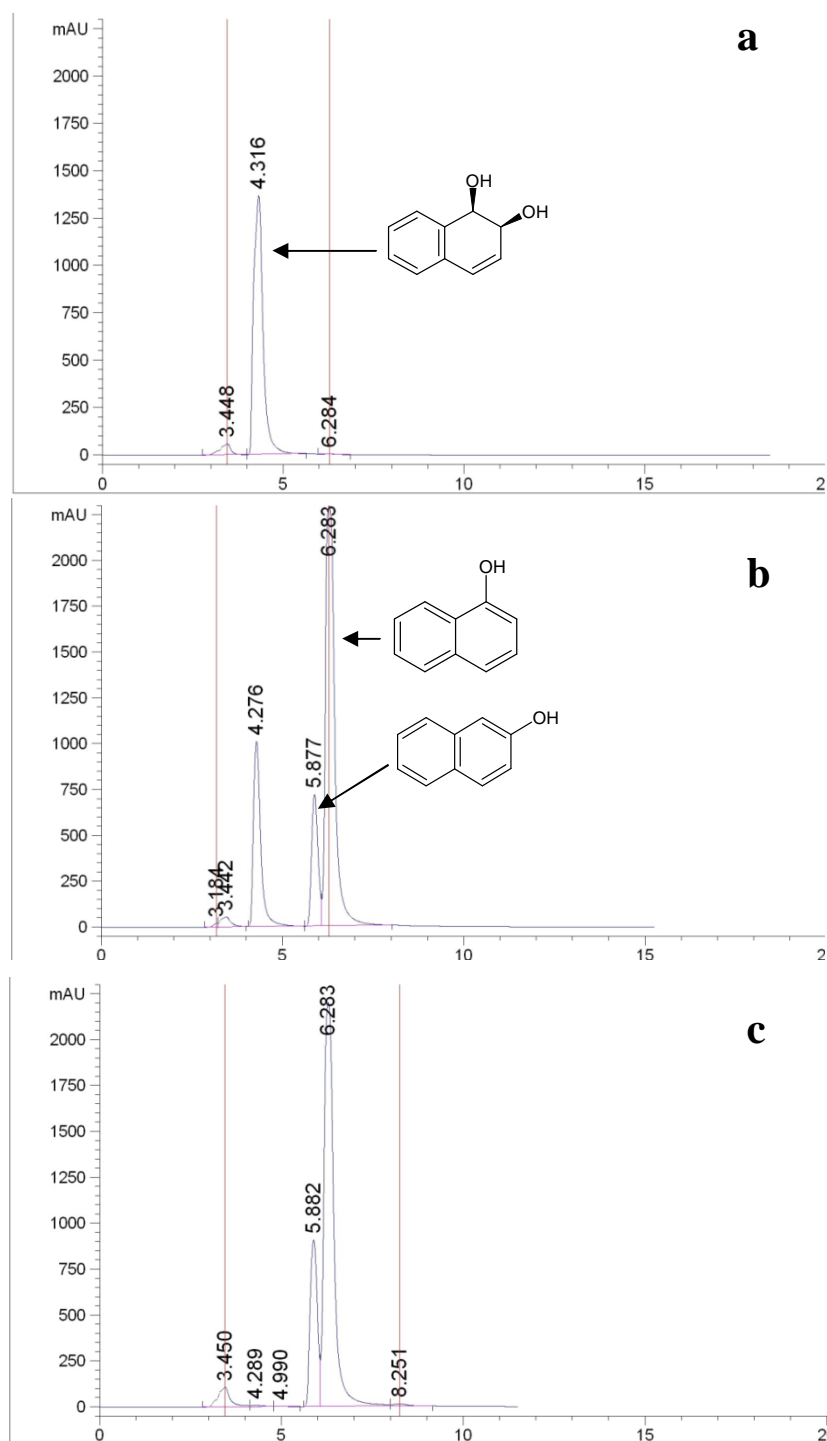


Fig. 4.15. Naphthalene biotransformation HPLC analysis. **a)** Chromatogram obtained from the biotransformation of naphthalene. **b)** Chromatogram of the sample after treatment with 35 μ l of H_2SO_4 and heated at 70°C for 15 minutes; peaks for 1- and 2-hydroxynaphthalene are visible. **c)** Chromatogram of the sample after treatment with a further 25 μ l of hot acid; the disappearance of the dihydrodiol peak can be seen.

Table 4.11. NDO biotransformation yields estimated by HPLC at 24 hours. Data are expressed in nmol/(mg protein).

Substrate/Product	Yield	Relative
naphthalene/ dihydroxynaphthalene	5355.9 ± 184.6	1
anisole/ phenol	1356.8 ± 76.6	0.25
benzophaeone/ hydroxybenzophaeone	387.7 ± 45.6	0.07
biphenyl/ dihydroxybiphenyl	281.8 ± 15.2	0.05
quinoline/ hydroxyquinoline	203.3 ± 28.8	0.04
1,2,3-trimethoxybenzene/ dimethoxyphenol	182.4 ± 28.0	0.03

5. *Discussion*

This study aimed to evaluate the potential of bacterial oxygenase in biotransformation. For this purpose, the enzymes ToMO and NDO were used; the first has long been used by this laboratory and is well characterised, the second was cloned during the course of this work.

5.1. Cloning of new oxygenases and construction of the TouA mutants

A number of bacterial strains, previously isolated from PHA enrichment cultures, were used to clone new oxygenases. Among these, genes coding for a functional naphthalene dioxygenase (NDO) were cloned from *Pseudomonas* sp. N1, while genes potentially coding for the subunit of a phenanthrene dioxygenase (PhDO) were cloned from *Sphingobium* sp. PhS. These two enzymes show substantial differences in terms of the gene arrangement: NDO has an operon-type gene configuration whereas PhDO is encoded by genes that are dispersed within the genome of the microorganism.

During the past few decades, several NDO enzymes, isolated from different Pseudomonadaceae, have been extensively studied and well described. In the current literature, it has been reported that these enzyme complexes are capable of oxidising more than sixty compounds composed of one or more aromatic ring and containing different substituents [102, 103]. In this study, a variant NDO was cloned and expressed in the heterologous host *E.coli* JM109; preliminary functional assays (indigo tests) confirmed its functionality. The *in silico* analysis of the sequence encoding NDO revealed an amino acid similarity equal to or less than 85% compared to the sequences of other naphthalene oxygenase enzymes whose activity had been studied; a difference of 15 % in the amino acidic sequence was considered sufficient to suggest a difference in enzyme activity in terms of substrate range and catalytic efficiency. Additionally, in the literature, no information regarding the activity of NDO on the substrates used in this study is available. Based on these observations, it was decided to test the cloned enzyme activity using biotransformation assays.

In other work, cloning procedures of PhDO did not produce plasmids that expressed a functional enzyme. The fact that the genes coding for terminal hydroxylase subunits, and those encoding electron carriers, are not clustered forced a rather complex cloning strategy. The configuration of the plasmid appeared to be a determining factor for the purpose of

enzyme expression: the first transfer of the inserts from the pGEM series of vectors into those of the pET series did not improve expression, however, the transfer into pET of the sole copy of the genes encoding catalytic subunits (*bphA1aA2a*) resulted in a structure whose expression was clear (pNC4). In addition, the inclusion of an RBS above each gene encoding the electron transporter subunits (plasmids of the pEM series) favoured, apparently, the expression of a single bphA3 subunit. Preliminary biotransformation assays of phenanthrene showed no evidence of catalytic activity from the entire PhDO enzyme complex, as was also shown in the associated indigo tests. However, further assays using the hybrid expression system NDO/PhDO appeared to confirm the function of the electron transporters BphA3 and BphA4. Although the system expressing the sole terminal hydroxylase (NDO-H) (pEM14) proved to be functional (demonstrating, moreover, high promiscuity), the hybrid expression system, containing genes encoding both NDO-H and the electron transporters *bphA3* and *bphA4*, was the most efficient. Based on current data, the most plausible hypothesis to justify the absence of PhDO activity is that the correct assemblage between the electron transporter and catalytic subunits did not occur. However, it should not be ruled out that the current cloned catalytic subunits might be associated with different transporters in the parent strain or that they are non-functional vestiges of duplication or horizontal gene transfer events. In order to achieve a system that expresses a functional PhDO, further bioconversion assays need to be carried out using new plasmid configurations as well as cloning *Sphingobium* sp. PhS genes that encode other catalytic subunits.

5.1.1. ToMO and TouA mutants

One of the main objectives of this work was to test the ability of ToMO, and certain TouA mutants, to attack a number of aromatic substrates not belonging to the PAH group. ToMO was isolated from *Pseudomonas* sp. OX1 and belongs to the subfamily of toluene monooxygenase [60] that groups structurally similar enzyme systems but with, sometimes, different functional characteristics. It has long been known that this enzyme is very flexible in terms of substrate range; the possibility of obtaining functional variants has therefore attracted the interest of many research groups since a number of products of its catalytic activity are molecules frequently used in biomedical and industrial fields. Following *in-silico* structural analysis and docking simulations carried out on ToMO, two mutants of the subunit TouA, D211A and D211A/E214G, were designed and produced. These mutations

affect the openings of the substrates' access channels and were introduced in order to facilitate the entry of substrates having a greater steric footprint compared to that of the natural substrate. The *in-silico* analyses showed that the D221A substitution causes an enlargement of one of the openings, while the D211A/E214G substitution leads to the fusion of both openings thereby generating a single, large opening. The mutations were made taking into particular account the effects of the steric footprint of the side chains of the two residues in positions D211 and E214 [71]; in the case of the substitution in position D211, it was decided to introduce the residue A, which has a low probability of altering the original secondary structure (α helix), due to its high α propensity. However, the fact that residues were replaced by apolar amino acids could, somehow, influence the transit of substrates bearing charged or polarised groups. It should also be considered that the predictions of the above-described *in-silico* analyses are subject to error, resulting from the fact that the software used, due to inherent limitations, do not consider the potential conformational variations that the structure of the TouA subunit can undergo following the introduction of such mutations; although all due caution was taken, it cannot be ruled out that, in natural physiological conditions, this could actually happen.

5.2. Biotransformations

5.2.1. Optimisation of the biotransformation protocol

In order to achieve a good biotransformation yield, the main steps of the protocol, cellular growth, induction and administration of the substrate, must be optimised; in this part of the study, the above conditions were focused on in order to obtain satisfactory biotransformation yields, from both *T_oMO* and NDO, for each substrate used.

Cell growth conditions were developed taking into account the needs of the various bacterial strains and in consideration of the fact that the medium of choice for the biotransformations was M9 mineral medium as it was devoid of components that could generate a high level of noise during analysis. Despite the heterologous host being the same (*E. coli* JM109), while the strains bearing constructs encoding NDO, *T_oMO* and E214G showed good growth in M9 minimal medium, those carrying constructs encoding D211A and D211A/E214G showed good growth only in LB medium. It was, therefore, decided to cultivate both strains in LB medium and, in order to maintain equal

experimental conditions and obtain comparable data, the same was done for the E214G mutant and for the wild type *ToMO*.

The induction process was developed using the indigo assay, a compound that is recognised and transformed into a radical indoxyl by many oxygenases, which spontaneously dimerises to form indigo. By setting up qualitative colorimetric tests, it was possible to determine the best possible induction time for both *ToMO* and NDO. The D211A and D211A/E214G mutants responded in different ways in this stage also, probably for the same reasons that made the optimisation of the growth stage problematic. However, an induction time of 120 minutes was chosen as this was considered to be the most reliable.

As the best characterised enzyme of those considered in this study, *ToMO* was chosen to optimise the biotransformation conditions for the various substrates. The substrates used in the biotransformation assays in this study were 1,2,3-trimethoxybenzene, anisole, benzophenone, bibenzyl, biphenyl, nitrobenzene, quinoline and trans-stilbene. These compounds were chosen because they have never previously been tested as substrates for *ToMO* and NDO, and also due to the important role that their hydroxylated products play in environmental, pharmacological and biotechnological applications. For example, biphenyl is the precursor of PCBs (polychlorobiphenyls), which are important environmental pollutants: oxygenases can catalyse the early stages of biphenyl breakdown. 2-Hydroxybiphenyl, known as Dowicide™, is applied on both an industrial and an agricultural level as a disinfectant and fungicide [<http://pubchem.ncbi.nlm.nih.gov>]. Bibenzyl forms the central core of stilbenoids, a class of biological active compounds whose hydroxylation could be an interesting biotechnological goal. *Trans*-stilbene and benzophenone, are compounds of certain biotechnological interest: mono- and di-hydroxy-trans-stilbene are precursors of resveratrol, a powerful antioxidant, while mono- and di-hydroxy benzophenone are precursors of photoprotective molecules [104]. Nitrophenols, nitrobenzene hydroxylated derivatives, are precursors of tolcapone and entacapone, two drugs used in the treatment of Parkinson's disease [105, 106, 107]. 3,4,5-Trimethoxyphenol, a 1,2,3 trimethoxybenzene monohydroxylated derivative, is a precursor of 2-(3-methyl-2-butenyl)-3,4,5-trimethoxyphenol, a potent anti-invasive agent used against solid tumors [108]. Quinoline is an example of a heterocyclic aromatic molecule and it would be interesting to ascertain the oxidation potential of oxygenases on this compound. In addition, 8-hydroxyquinoline is a disinfectant with a wide range of antifungal actions [<http://pubchem.ncbi.nlm.nih.gov>].

Each of the compounds described above has particular chemico-physical characteristics and a certain degree of dose-dependant cytotoxicity; so, in order to ensure the maximum biotransformation efficiency, a specific protocol for administering the substrate to the cellular suspension in the water phase should be optimised for each compound. Given the, partly exploratory, nature of the objectives of this study, and considering that these molecules can regroup into three classes on the basis of certain of their physical characteristics (1: liquid, volatile and water-insoluble, 2: water-soluble and 3: solid and water-insoluble), it was only possible to standardise three methods of administration using the compounds toluene, nitrobenzene and biphenyl, each one representative of one of the three physical classes stated above.

Biotransformation experiments carried out on toluene, the only compound belonging to Class 1, above, showed that administering the substrate in a vapour phase (saturating conditions) gives the best yields. The biphasic administration method, although functional, did not provide a yield equal to that described above. This was probably due to the strong tendency of the organic phase to sequester toluene; the use of an organic phase containing a higher concentration of substrate compared to that tested could have, in theory, improved the biotransformation yield. Although, for the purposes of this research, both the protocols provided acceptable yields, it was decided to carry out subsequent biotransformations using the most efficient method.

As nitrobenzene is water soluble, it was added directly to the water phase. The biotransformation yield was evaluated using HPLC, by monitoring the reduction in the amount of substrate and was found to be satisfactory. The quantity of product formed could not be measured because no HPLC signal corresponding to any of the expected nitrophenols was observed. However, the obtained signal, compatible with that of a nitrocatechol, which is unavailable as a commercial standard, increased with the decreasing signal of nitrobenzene, suggesting that the signal was the result of the ToMO activity [60]. Given its good yield and the failure of potentially interesting alternative methods, this method was chosen to administer the substrate to subsequent bioconversion assays of water soluble substrates.

The biphenyl biotransformation was carried out under three different conditions: using a biphasic system; in the presence of DMSO; and by directly administering the substrate to the water phase in a pure form. The much higher yield of the latter method is probably attributable to the maximised water solubility of the substrate and to the fact that no other compounds capable of interfering with the cellular activity are present. In fact DMSO, that,

thanks to its permeabilising effects on cell membranes, and its ability to produce in solution a higher quantity of substrate than thought, could have increased the reaction yield, has shown a damaging aspect. Even the biphasic system did not produce such high biotransformation yields; the cause of this low efficiency could be due to the high K_{ow} of the substrate that, in the concentrations used, could be poorly bioavailable.

5.2.2. Biotransformation results

ToMO and TouA mutants

ToMO proved capable of hydroxylating, albeit with varying efficiency, all the substrates tested which confirmed its great versatility in terms of substrate range and also its low regiopreference. The same can be said for the reference mutant E214G but not for the D122A and D211A/E214G mutants, which were unable to attack many of the molecules tested. For each substrate tested, the isomer mixes produced by all the ToMO variants were similar in composition, with the exception of the E214G mutant in the biotransformation of 1,2,3-trimethoxybenzene, where the predominant isomer was 3,4,5-trimethoxyphenol; here, the E214G mutant seemed able to influence the enzyme regiopreference, probably favouring a particular orientation of the substrate at the entrance of the channel leading to the active site. These findings assume that the enzyme is affected by the electrostatic effects of the substituents on the aromatic ring of the substrate. Chemically, it is well known that, in the presence of activating groups, the electrophilic aromatic substitution on substituted aromatic rings happens in the *ortho/para* position while in deactivating substituents it happens in the *meta* position. This behaviour was also found in biotransformations: when molecules with activating groups were used (a methoxyl group in anisol, a phenyl group in biphenyl, and an ethylbenzene group in bibenzyl), the *para* isomer was predominantly produced. Conversely, biotransformations using molecules with disactivating groups (a nitro group in nitrobenzene, a ketone group in benzophenone and a vinylbenzene group in *trans*-stilbene), gave the *meta* isomer as the major product. An interesting result was that relating to the biotransformation of quinoline, which was oxidised by both the wild type and the E214G mutant at the level of the heteroatom; this was the only case in which the enzyme showed a high regiospecific reaction.

Surprisingly, neither the D211A nor the D211A/E214G mutants were able to hydroxylate molecules larger than natural substrates, such as nitrobenzene, quinoline and *trans*-stilbene; however the D211A/E214G mutant, unlike D211A, was able to hydroxylate

bibenzyl. Obviously, these results contradict the *in-silico* model, according to which the inserted mutations should have favoured enzyme activity on substrates that are actually larger than the natural compounds. This unexpected behaviour could be due to the fact that the *in-silico* model has inherent limitations: the model is in fact based on a static structural datum, the crystallographic structure of ToMO, and is not able to predict how the substitutions can impact on the secondary and tertiary structure of the TouA subunit. Therefore, although the choice of the substitutions was made from the perspective of minimising the distortion risk of TouA, it cannot be ruled out that the introduced mutations caused unpredictable conformational variations, causing dissimilarities to the enzyme structure that differed to those predicted by the model. Alternatively, additional and undesirable mutations when the mutants are being created could explain this unexpected behaviour: the mutants were produced using site directed mutagenesis performed on a plasmid containing the entire cluster *tou* (6 kb) following which only the section of DNA affected by mutations, of about 800 bp, was checked by sequencing. Therefore, it is possible that other mutations were introduced to the remaining encoding sections that were able to alter the enzyme function. However, in the light of these results and of the anomalous behaviour in the growth phases described above, it is not possible to express a definitive judgement regarding the activity of the D211A and D211A/E214G mutants. One possibility of overcoming many of the above doubts, consists of reproducing the constructs expressing the mutants by excising a small mutagenesised piece of the plasmids used so far and reinserting the same into pMZ1256, which contains the entire gene cluster coding for the wild type enzyme.

Quantitative analyses were carried out only for biotransformations where a commercial standard was available. In the case of the toluene biotransformation, the experimental conditions adopted allowed the specific activity of each ToMO variant to be calculated. In accordance with the current literature [71], the wild type and E214G were found to have a very similar specific activity and, in addition, this activity was far greater than that of other ToMO variants. The specific activity of D211A was similar to that of D211A/E214G. In all the toluene biotransformations performed using the ToMO variants, the reaction was seen to slow down significantly to around 60 minutes.

With regard to the biotransformation of all the other substrates, for which it was possible to carry out a quantitative analysis, the E214G mutant was found to be the most efficient, followed by the wild type. Except for the benzophenone biotransformation by the D211A mutant, which was found to be only slightly superior than the wild-type in terms of yield

after 24 hours, both the D211A and the D211A/E214G mutants showed, for each substrate, a lower biotransformation yield after 24 hours than that of the wt. At first, these results suggest that the introduced mutations have reduced the catalytic efficiency of the enzyme, however, in view of the assumptions previously made regarding the failure of the mutants to function on certain substrates, these results cannot be considered definitive. The fact that the wt enzyme, on certain substrates, showed higher yields after 24 hours to those obtained with toluene (Fig. 4.11), does not necessarily mean that the wt has a higher specific activity; only by studying the enzyme kinetics of each substrate can the correct conclusions regarding relative specific activities be drawn.

NDO

GC-MS analysis showed that, according to the substrate used, NDO is able to cause three different types of reaction: dealkylation (1,2,3-trimethoxybenzene and anisole), monohydroxylation (quinoline), and dihydroxylation (naphthalene, benzophenone, bibenzyl, and biphenyl). Biotransformation products were not observed for nitrobenzene and *trans*-stilbene. The regiopreference of the enzyme varies according to the substrate used: in the case of naphthalene and benzophenone, the enzyme showed a distinct regiospecificity while the biotransformations of biphenyl, bibenzyl and quinoline produced mixtures of isomers.

Naphthalene was used as a reference substrate and its biotransformation produced a mixture of different compounds: the expected dihydroxylated compound 1,2-dihydroxy-1,2-dihydro-naphthalene, and the two monohydroxylated compounds 1-naphthol and 2-naphthol. Dihydroxylated compounds such as 1,2-dihydroxy-1,2-dihydro-naphthalene tend to restore aromaticity, using spontaneous dehydration, thus giving rise to a mixture of monohydroxylated derivatives; the probability of losing one of the two OH groups is identical, therefore the resulting mixture is composed of equal quantities of each monohydroxylated compound. In the biotransformations of naphthalene, biphenyl and benzophenone, for which the related dihydrodiols were not commercially available, the tendency of these compounds to restore the aromaticity was exploited in order to carry out an indirect quantification of the product: using a mild treatment with H₂SO₄ and subsequent heating, the dihydrodiols present were converted into monohydroxylated compounds, which were subsequently quantified using HPLC.

The bioconversion yields after 24 hours, with the exception of bibenzyl and *trans*-stilbene, for which the relative mono- and dihydroxylated compounds were not

commercially available, were determined for each substrate. Overall, the activity of NDO on the tested substrates was significantly lower than that on its natural substrates: the apparent tendency was that the activity of the enzyme normally diminished with increasing steric footprint of the substrate. In fact, anisole, a relatively small substrate, is converted with greater efficiency compared to other substrates. Despite the yields of biotransformations being rather low, there is evidence that NDO is able to recognise many of the substrates tested, which suggests that this enzyme could be a good candidate for other experimental approaches, for example, the construction of NDO mutants aimed at improving the catalytic efficiency of NDO on these substrates.

*6. Internship at “Water Research Institute”
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Supervisors:

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Maximino Manzanera Ruiz Ph.D.

Looking for *Halomonas variabilis* W10's genome regulatory elements involved in the polycyclic aromatic hydrocarbons breakdown: a new “flow cytometry based” screening method.

Background

Polycyclic Aromatic Hydrocarbons (PAHs) represent important crude oil derived water pollutants. These compounds can affect pelagic and coastal marine environments as a result of industrial activity and catastrophic events. In 2002 the Prestige oil tanker accident caused the release of a great quantity of fuel oil that polluted thousand of kilometres of Spanish and French coastline. Seawater and sediment samples were collected by Repsol YPF from the area surrounding the wreck, at a depth of 4000 m along with fuel samples from one of the tanker's fuel containers. From these samples, twenty one bacterial strains with the ability to degrade hydrocarbons and/or produce useful bioemulsifiers were selected [109]. One of the isolated strains was identified as *Halomonas variabilis*, then called *Halomonas* v. W10: this strain exhibited the ability to use naphthalene and other PHAs as a sole carbon and energy source. Additionally, compared to other isolated strains, it was able to produce the highest amount of efficient biopolymers with emulsifying activity. In order to look for regulatory and structural genes involved in degradation of PHAs and bioemulsifier production, a *Halomonas variabilis* W10 genome library was constructed, using a p18GFP operon trap vector and the heterologous host *Escherichia coli*.

Aims

The aim of this study was to develop a new protocol for bacterial genome library screening using a Substrate Induced Gene EXpression (SIGEX)-based method [87]. Naphthalene, a substrate that *H. variabilis* W10 is able to use efficiently as a sole carbon and energy source, was chosen as the pilot PHA inducer to optimise this protocol.

Methods and materials

Cultures:

E. coli cultures were prepared using Tryptic Soy Broth (TSB) or agar (TSA) medium (DIFCO).

Additions: Ampicillin (Ap) at a final concentration of 0.1 mg/ml.

Culture induction:

Naphthalene (SIGMA-ALDRICH), used as a PAH inducer, was added to the TSB medium at a concentration of 5 mg/ml. The solution was incubated for 48 hours at 200 r.p.m. before use, in order to allow maximum naphthalene solubilisation.

Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the medium at a final concentration of 1 mM.

Flow cytofluorometric and fluorometric analyses

Flow cytofluorometric analyses were performed by a BD FACSAria™ III flow cytometer.

Fluorometric analyses were performed by a FLUOstar OPTIMA microplate fluorometer (BMG-LABTECH).

Results

In this study the SIGEX-based method was adapted to screen a previously prepared *Halomonas variabilis* W10 genome library. The library was prepared in liquid culture using the *E. coli* heterologous host. The library cell concentration was 10^7 CFU/ml (O.D.₆₀₀ = 0.6), the optimal value for flow cytofluorimetric analyses.

Sorting of GFP non-expressing (GFP-) E. coli cells

Using the *Halomonas variabilis* W10 genome library, two library cultures were prepared: two test tubes, containing 4 ml of TSB + Ap and 4 ml of TSB + Ap + IPTG respectively, were each inoculated with 1 ml of library culture. Both tubes were incubated at 37 °C for 1 hour at 200 rpm, which were appropriate conditions to maintain the 10^7 CFU/ml cellular concentration and to guarantee a good induction. Then, cultures were analysed by flow cytometry; in the induced sample, flow cytofluorometric analysis revealed the presence of a high percentage of GFP expressing (GFP+) cells (69.2 %) (Fig. 1a); in the sample without the inducer, the GFP+ cell percentage was very low (6.6%) (Fig. 1b). In the induced sample, flow cytofluorometric sorting (Fig. 2) reduced the initial cell concentration to 10^5 CFU/ml, so it was necessary to concentrate the sample, by centrifugation to 10^6 CFU/ml.

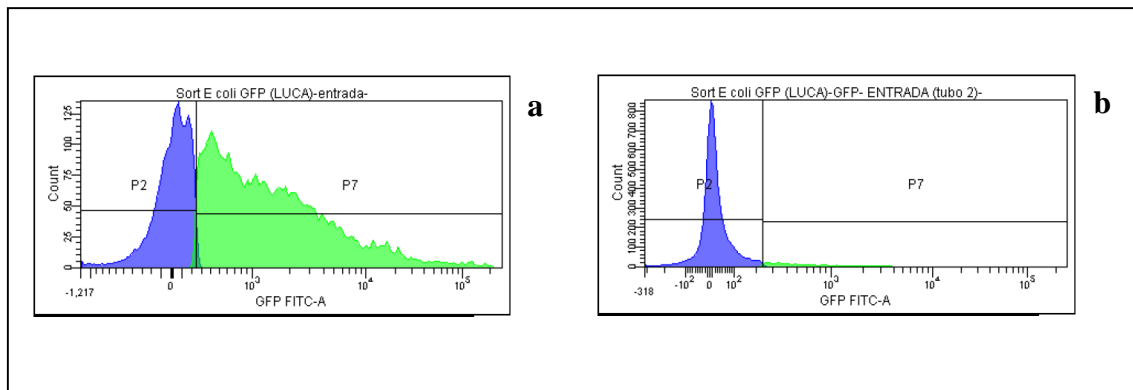


Fig. 1. IPTG induced (a) and non-induced (b) library cytofluorometric analyses. The green areas represent the percentage of GFP+ cells, the blue areas represent the percentage of GFP- cells.

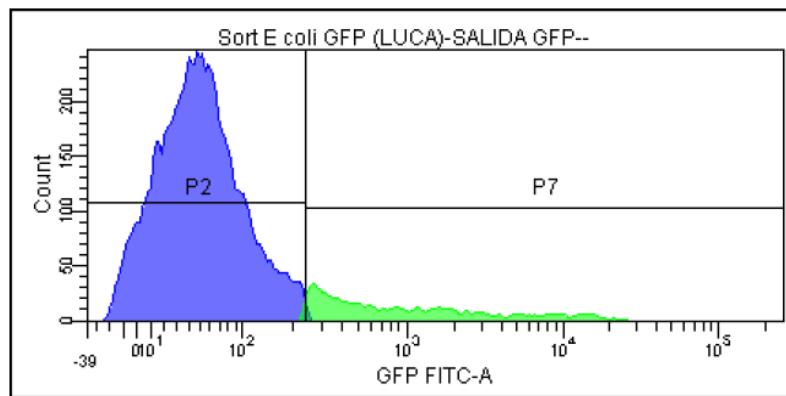


Fig. 2. Sorted GFP- cells cytofluorometric analysis. The green area represents the percentage of GFP+ cells, the blue area represents the percentage of GFP- cells.

Sorting of GFP+ E.coli cells

Four library cultures were prepared by adding 100 μ l of GFP- culture to 5 ml of TSB medium; culture induction conditions are shown in Table 1. The cultures were incubated at 37°C for 4 hours at 200 r.p.m. Each culture was analyzed by flow cytometry: Figure 3a shows that the naphthalene/IPTG induced sample (Culture 1) comprised a 15% presence of GFP+ cells; as expected. Control samples 1 and 2 contained very low levels of GFP+ cells (Fig. 3c, 3d) and in the negative control no GFP+ cells were observed (Fig. 3b). GFP+ cells were sorted from the naphthalene/IPTG induced sample; however, the control cytofluorimetric analysis of these cells showed a 20% presence of GFP- cells (Fig. 4). The

sorted GFP⁺ cells were then concentrated by centrifugation to 10³ CFU/ml in a total volume of 1 ml.

Table 1. Induction condition for the cytofluorometric sorting of GFP⁺ cells.

	IPTG	Naphthalene	Notes
Culture 1	+	+	Sample
Culture 2	+	-	Control 1
Culture 3	-	+	Control 2
Culture 4	-	-	Negative control

^a **Symbols:** +, presence of inducer; -, absence of inducer.

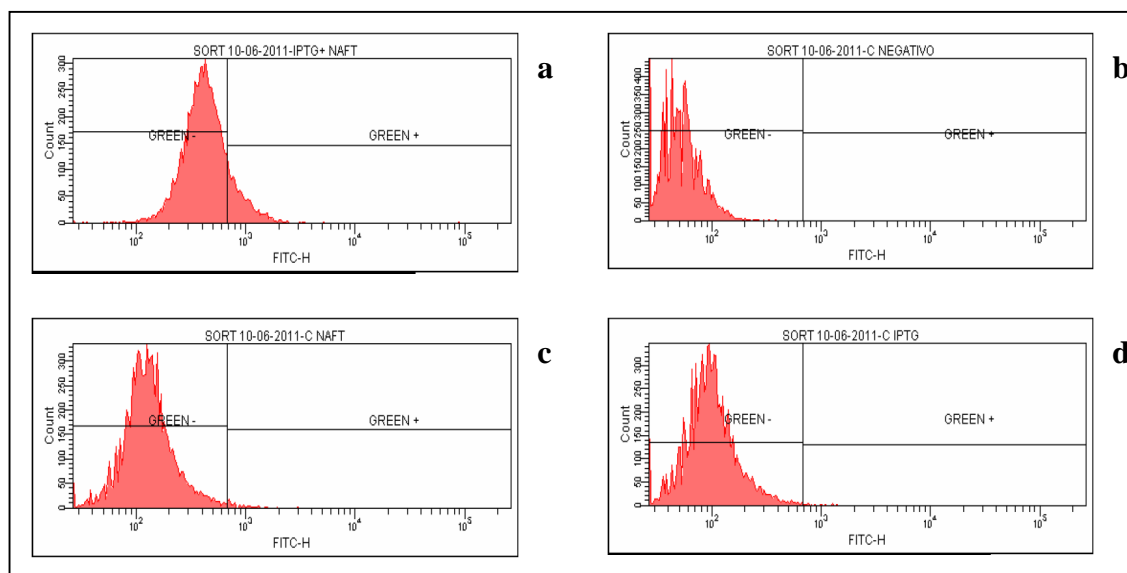


Fig. 3. Cytofluorometric analysis of cultures 1 (a), 2 (c), 3 (d) and 4 (b). The areas in the green + field represent the percentage of GFP⁺ cells present in the sample.

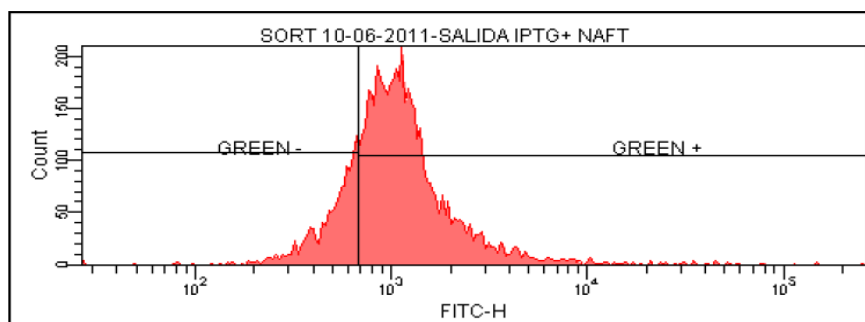


Fig. 4. Cytofluorometric analysis of sorted GFP⁺ cells. The area in the green + field represents the percentage of GFP⁺ cells present in the sample.

Fluorometric analyses

To isolate the GFP+ clones, all the previously obtained GFP+ cell suspensions were plated on 9 TSA plates. After 24 hours of incubation each plate showed 60 – 70 colonies, some of which (approximately 30%) displayed a green colour. Each of the 650 colonies was collected by a sterile capillary and, using 96-well microplates, dissolved in 100 μ l of TSB (TSB + Ap) medium, according to the schematic shown in Figure 5. In each well, the cellular concentration was approximately 10⁸ CFU/ml. As a negative and a positive control, an *E. coli* strain and a *E. coli* strain constitutively expressing GFP were used, respectively. To obtain cells with an adequate metabolic status for the induction, using the first microplate set (comprising 11 plates) a second microplate set was prepared: from each first-set well, 10 μ l of culture were transferred to a new microplate well containing 90 μ l of TSB (TSB + Ap) to obtain a 1:10 dilution. These microplates were incubated at 37°C for 3 hours at 200 r.p.m. Following incubation, the resulting cellular concentration was 10⁸ CFU/ml.

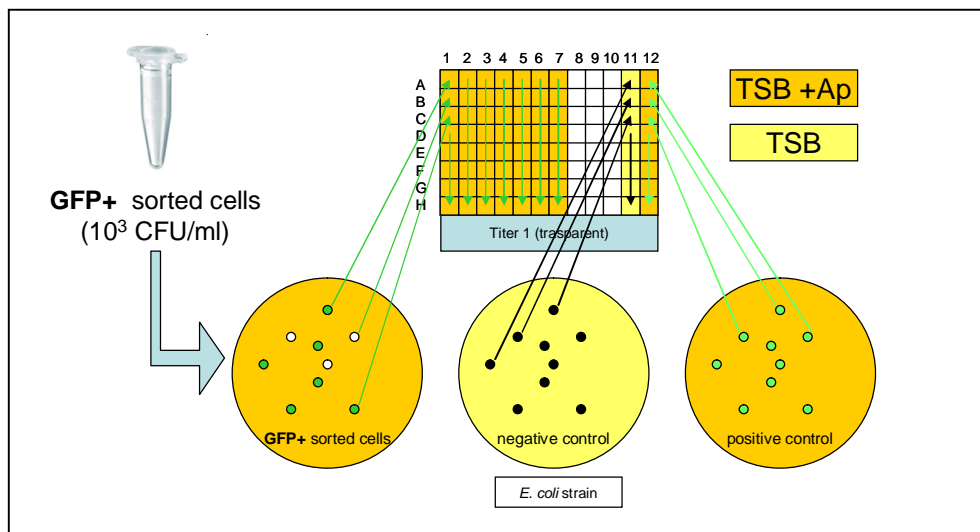


Fig. 5. Microplate (1st set) preparation schematic.

As shown in Figure 6, from each second set microplate, 10 μ l of culture from each well were subsequently transferred to four new microplates, which were prepared by adding 90 μ l of TSB (TSB + Ap) to each well. In this step, to avoid the green light scattering between adjoining wells during fluorometric analysis, black 96-well microplates were used. The four black microplates (one for the sample and the others for the controls) were prepared with the same induction conditions that were used for the GFP+ cell sorting, and were

incubated at 37°C for 4 hours at 200 r.p.m. Fluorometric analyses was performed on each microplate using an excitation wavelength of 485 nm; the emitted radiation at 530 nm was registered and compared in intensity with that emitted from the control samples. This approach allowed 60 GFP positive clones to be isolated from the sample microplates. . From each of the 60 GFP positive wells, 50 µl of culture were collected to inoculate 60 tubes, each containing 3 ml of TSB + Ap. The cultures were incubated at 37°C for 24 hours and subsequently used to prepare a glycerol stock.

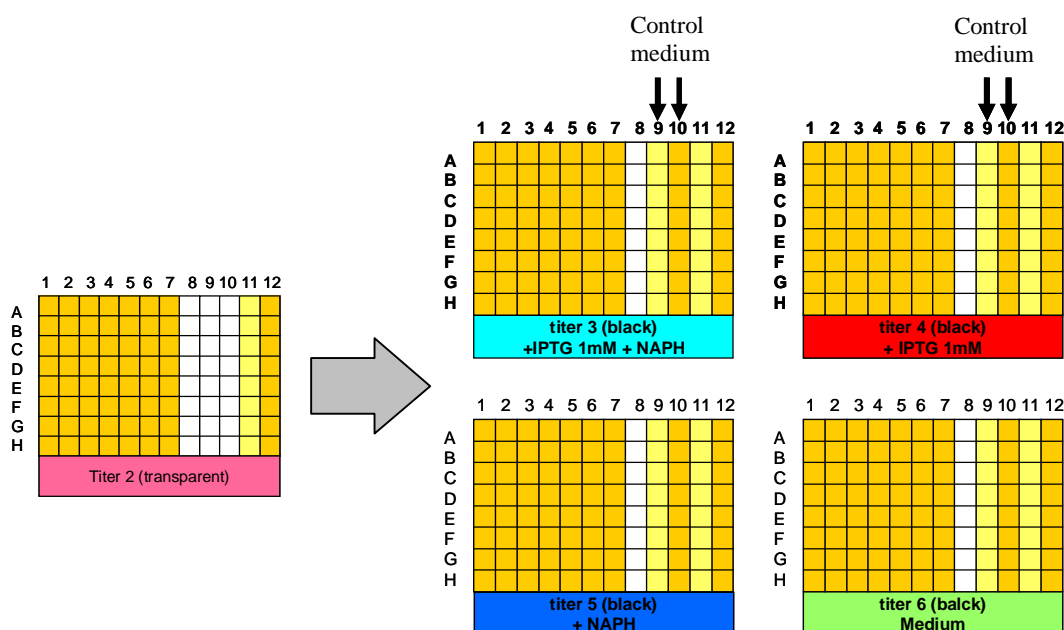


Fig. 6. Microplates prepared from the 2nd microplate set (titer 2). Titer 3: sample microplate; titer 4, 5 and 6: control microplates.

Discussion

In this study the SIGEX method, developed for screening metagenomic libraries, was adapted to screen a single bacterial genome library. The flow cytometry used was suitable for eukaryotic cell analysis, which explains the presence of a significant percentage of undesired GFP⁺ and GFP⁻ cells in the sorted samples (Fig. 2; Fig. 4). Furthermore, during the protocol optimisation phase, it was apparent that flow cytometric sorting affects cell vitality; in fact, if the sorted cells were not used immediately for the subsequent step, the resulting cellular growth was compromised. For this reason, following GFP⁻ cell sorting, it is recommended that the GFP⁺ cells be sorted within a short time and the GFP⁺ sorted cells be plated immediately. As described above,

positive clones can be identified by analysis of fluorometric data. In this study the GFP+ clones were selected by an approximate comparison of the observed values with those of the controls; only approximately 10% of the analysed clones were GFP positive, however, by using a statistic-based selection method, this value could be increased. To fix a standardised and reliable selection method (a confidence interval), statistical analysis of new fluorometric data should be performed.

Overall the results suggested that, compared to traditional library screening methods, this approach allowed a satisfactory number of cells to be selected that had a high probability of containing a plasmid carrying PHA-dependent regulatory elements thus saving time, materials and human resources.

Sequencing the 60 isolated clones with a high probability of containing a naphthalene-dependent regulatory element could allow this new screening method to be validated.

7. Conclusions

The bacterial oxygenases are extremely versatile enzymes and are of great use in a number of applications in environmental, biotechnological and biomedical fields. These biocatalysts have attracted the interest of many research groups around the world, and the methods used to study them are constantly evolving; traditional techniques are now combined with molecular and computational methods and, additionally, even more powerful approaches based on advanced screening techniques.

In this study, five important objectives were achieved:

- 1) a new naphthalene dioxygenase was cloned from *Pseudomonas* sp. N1 and characterised at a functional level to test its activity on aromatic substrates that had never previously been tested. The results of the biotransformations showed that this enzyme, albeit with low efficiency, is capable of recognising and attacking many of the substrates tested and, therefore, appears to be a prime candidate for further molecular studies.
- 2) Certain subunits of a phenanthrene dioxygenase were cloned from *Sphingobium* sp. PhS and the functionality of a number of these (the electron transporters) was confirmed by a naphthalene bioconversion assay performed using a hybrid enzyme. This work has laid the foundations for a new avenue of research, which aims to clone the whole functional enzyme and characterise the same in terms of substrate range.
- 3) A number of biotransformation protocols were developed that are able to guarantee, for the various substrates used, yields of sufficient quantity to allow qualitative and quantitative analyses to be carried out. At this stage of optimisation, a few important conditions, such as cell growth and induction, have reached a satisfactory level of standardisation. However, in consideration of the different chemical and physical characteristics, a specific administration protocol should be developed for each substrate used. These protocols would provide an excellent starting point for any future refinements.
- 4) The toluene/o-xylene monooxygenase, long studied by this laboratory, was characterised at the functional level on substrates that had never before been tested with this enzyme. The results showed that ToMO was able to recognise and attack, albeit with less efficiency compared to the natural substrates, all the substrates used. These data, in addition to having demonstrated the great metabolic versatility of this enzyme, suggest that it has great potential in the field of bioconversion. Furthermore, two mutants, D211A and D211A/E214G, that had previously been produced in this laboratory, were characterised using the same substrates. These results, even though far from expectations, could provide a useful information base for the production of new functional mutants. Future molecular

approaches to could lead to the creation of ToMO variants that are more powerful in terms of catalytic efficiency and that exhibit a hydroxylation regiopreference that would be of use in the production of strains of a biotechnological interest.

5) During an internship at the University of Granada (Spain), a new protocol for screening bacterial genomic libraries was developed using the SIGEX method. In a relatively short time, sixty clones with a high probability of containing naphthalene-dependent regulatory elements were isolated. It is extremely probable that these regulatory elements are topologically close to the structural genes that encode the enzymes involved in naphthalene breakdown pathways. Therefore, as can easily be imagined, this technique could also be applied to the research of genes encoding new oxygenases. The sequencing of the sixty isolated clones is currently in progress and could confirm the effectiveness of this new method.

The results obtained in this work are not considered conclusive but do, however, open up new research prospects on the oxygenases, ToMO and NDO. These oxygenases have demonstrated a high potential in the field of biotransformation and it cannot be ruled out that further molecular characterisation studies could lead to obtaining new enzyme variants that are even more versatile and efficient.

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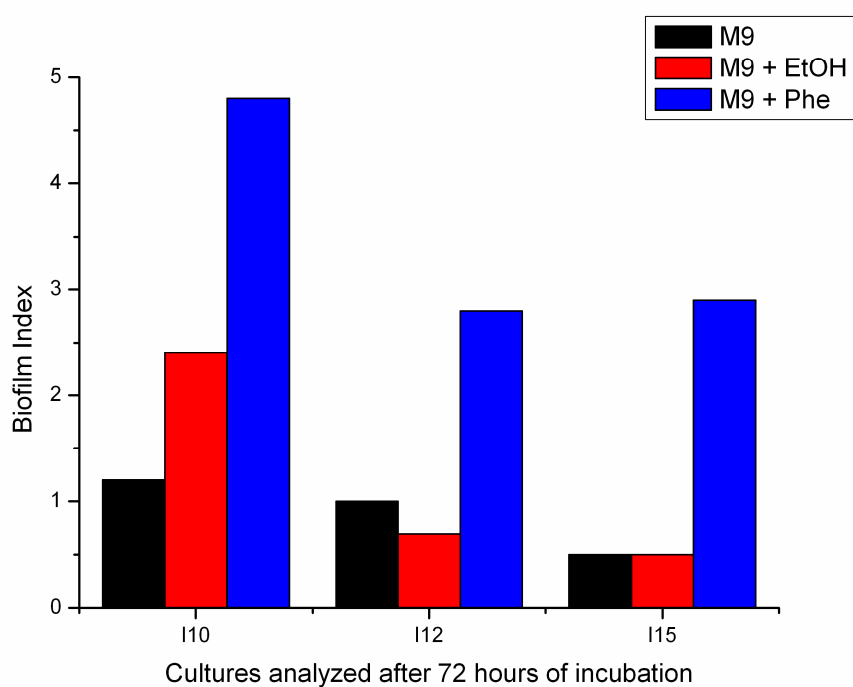
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Appendices

Appendix 4.1

The graph shows the BI values calculated for the I10, I12 and I15 strains after 72 hours of incubation in the presence of: M9 medium only (1st negative control); M9 + ethanol (2nd negative control); and of M9 + phenanthrene.



Appendix 4.2

N1 strain 16S rDNA sequence

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N2 strain 16S rDNA sequence

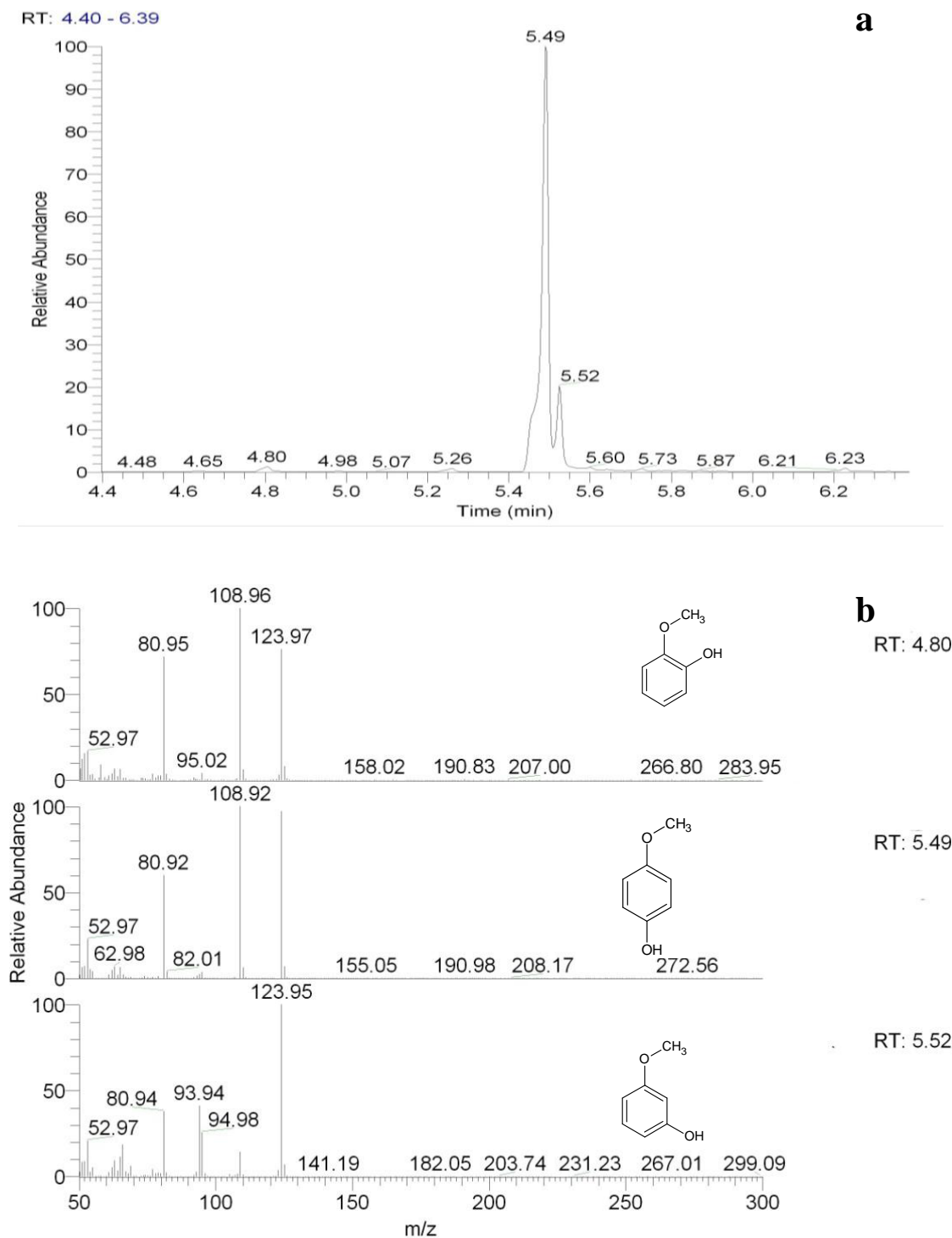
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Appendix 4.3

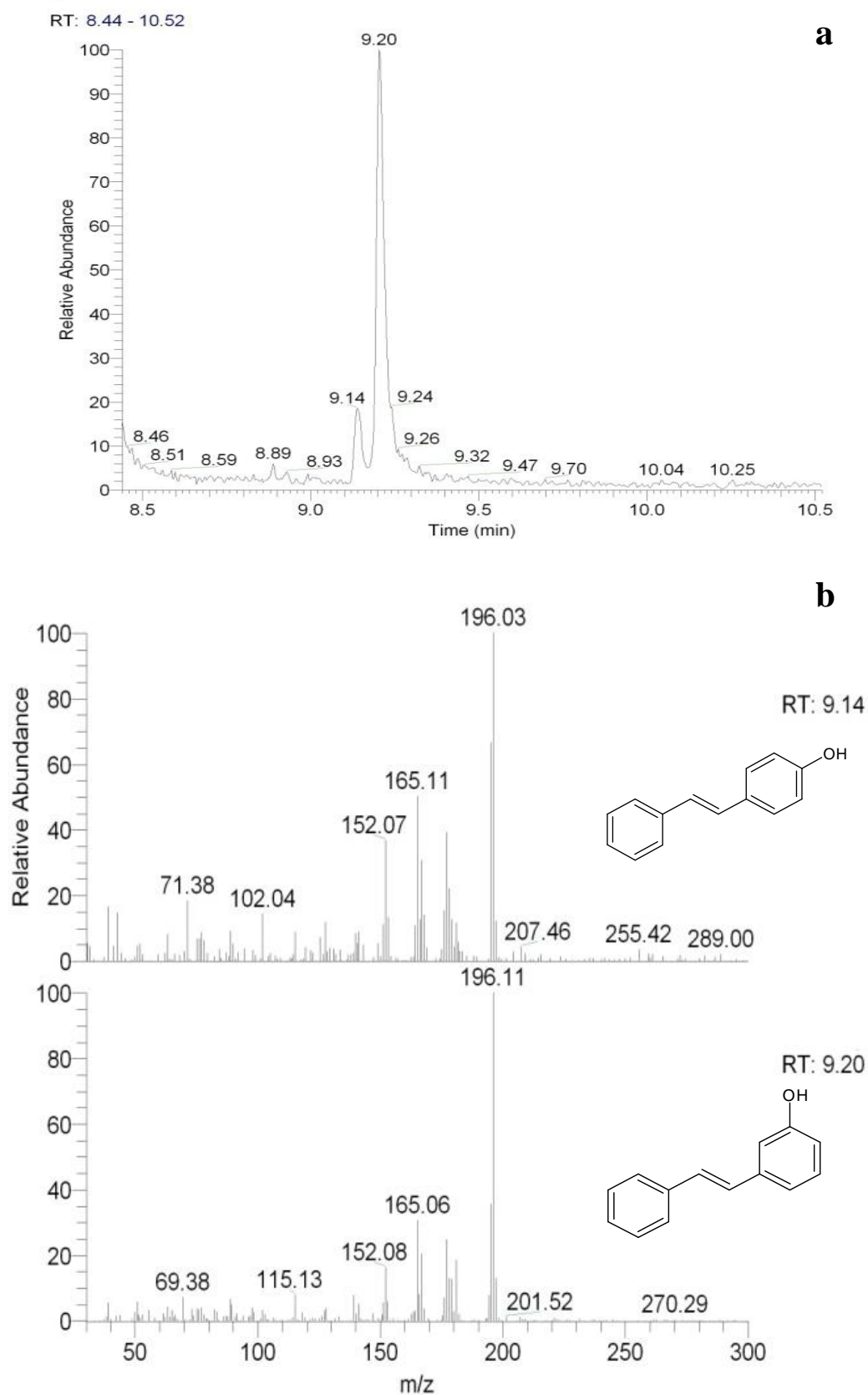
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Appendix 4.4

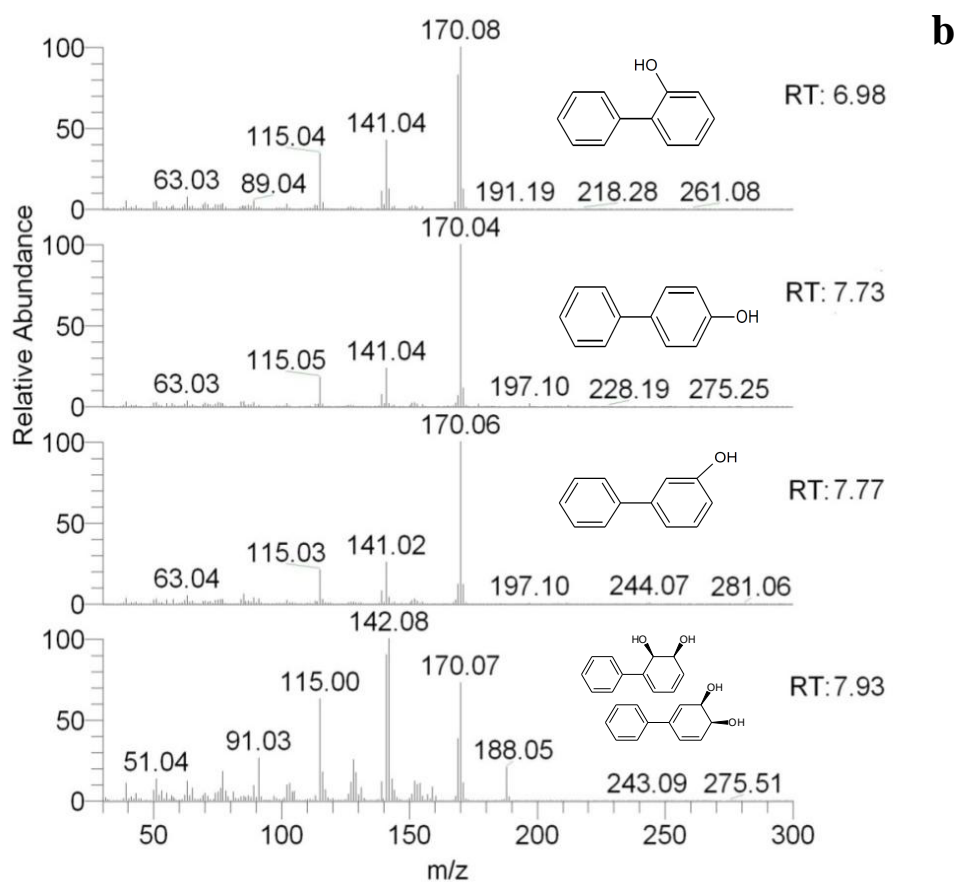
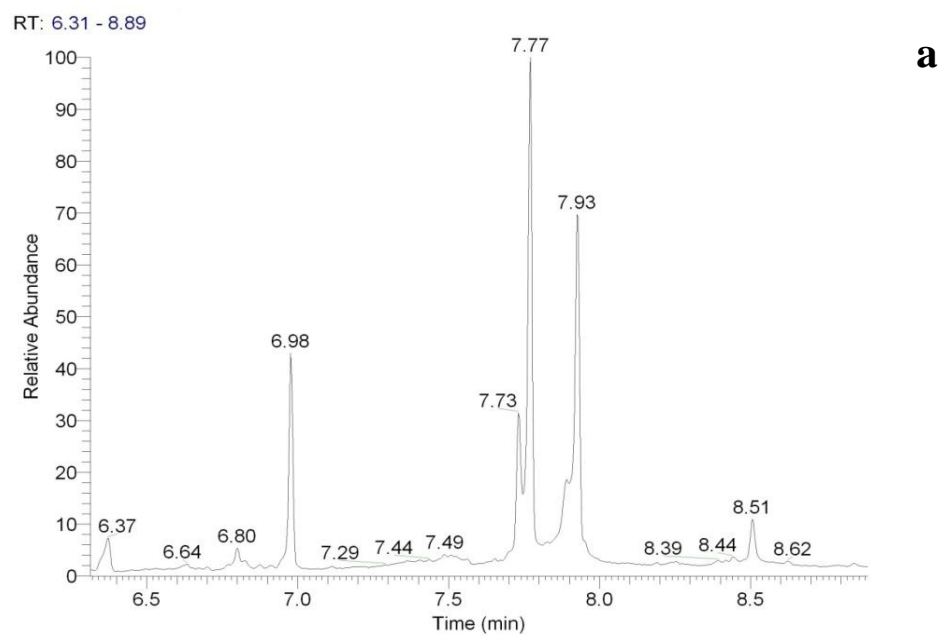


App. 4.4a. GC-MS analysis of anisole biotransformation product by ToMO. **a)** Chromatogram; **b)** mass spectra of: 2-methoxyphenol (R.T. 4.80), 4-methoxyphenol (R.T. 5.49) and 3-methoxyphenol (R.T. 5.52).

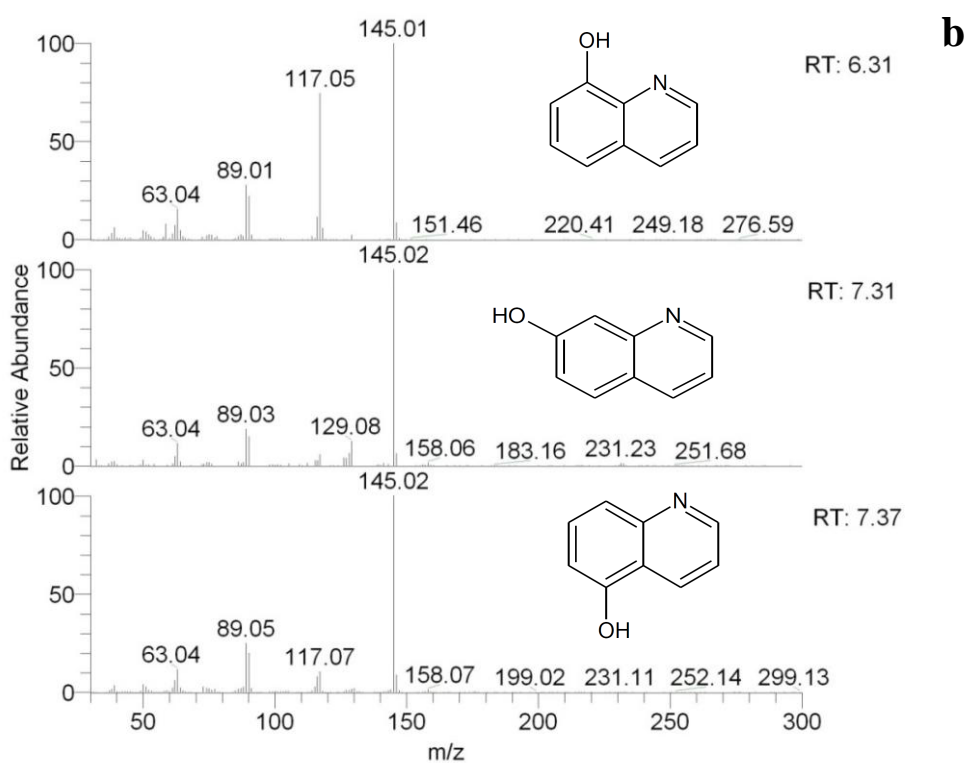
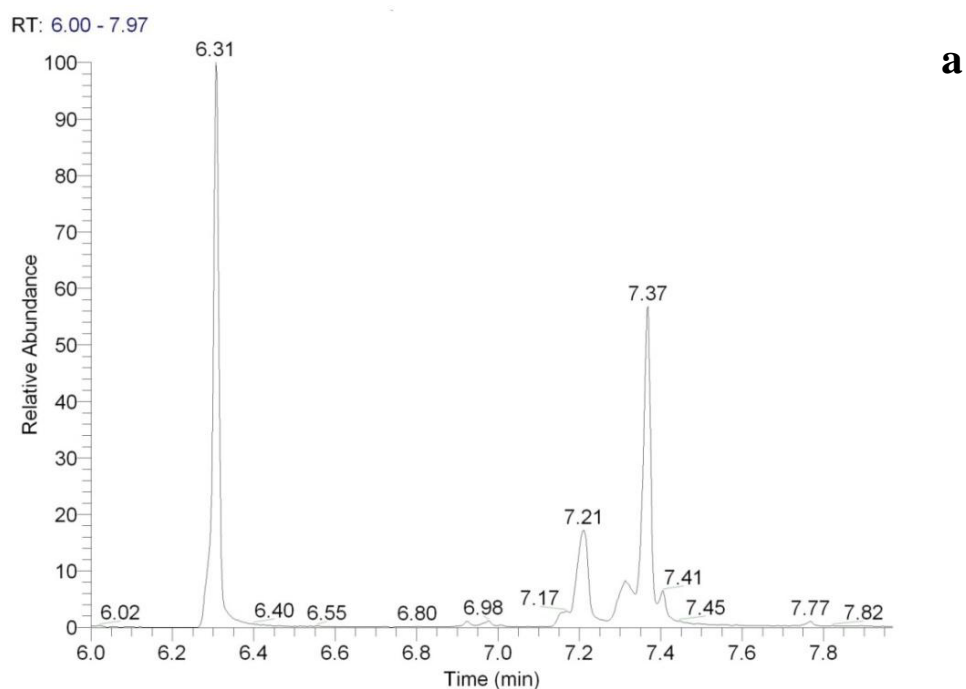


App. 4.4b. GC-MS analysis of *trans*-stilbene biotransformation product by ToMO. **a)** Chromatogram; **b)** mass spectra of: 4-hydroxy-*trans*-stilbene (R.T. 9.14) and 3-hydroxy-*trans*-stilbene (R.T. 9.20).

Appendix 4.5



App. 4.5.a. GC-MS analysis of biphenyl biotransformation products by NDO. **a)** Chromatogram; **b)** mass spectra of: 2- hydroxybiphenyl (R.T. 6.98), 4-hydroxybiphenyl (R.T. 7.73), 3-hydroxybiphenyl (R.T. 7.77), 1,2-dihydro-1,2-dihydroxybiphenyl and 2,3-dihydro-2,3-dihydroxybiphenyl (R.T. 7.93).



App. 4.5.b. GC-MS analysis of quinoline biotransformation products by NDO. **a)** Chromatogram; **b)** mass spectra of: 8-hydroxyquinoline (R.T. 6.31), 7-hydroxyquinoline (R.T. 7.31) and 5-hydroxyquinoline (R.T. 7.37).

Acknowledgements

I would like to thank my supervisor, Prof. Paola Barbieri, for her support and availability and highly valuable comments on drafts of this thesis.

I also thank Viviana Orlandi, Ivan Vaghi, Guido Sello (Department of Chemistry – University of Milano) and Patrizia Di Gennaro (Department of Biotechnology and Biosciences-University of Milano Bicocca) for their support and useful advise in this work.