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Baeyer-Villiger monooxygenases from a *Dietzia* sp.

Enzyme discovery, characterization and engineering

Serena Bisagni



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DOCTORAL DISSERTATION

by due permission of the Faculty of Engineering, Lund University, Sweden.

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Faculty opponent

Prof. Marco W. Fraaije, University of Groningen, The Netherlands

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| Abstract With the emergence of Green Chemistry, biocatalysis is becoming an important approach in many laboratory and industrial processes. Enzymes catalyse chemical reactions with high regio- and stereoselectivity at mild conditions and, most importantly, are able to form products that are not possible to obtain by conventional synthetic chemistry. Monooxygenases are a fascinating group of enzymes which oxidise substrates using atmospheric oxygen and release water as a by-product. These enzymes have great potential applications and indeed there are already some industrial showcases. Monooxygenases are of different types and one of the important groups is known as Baeyer-Villiger monooxygenases (BVMOs). BVMOs oxidise ketones to esters and heteroatoms to the corresponding oxide, which are interesting reactions for pharmaceutical and fine chemical industry. To date, several BVMOs have been discovered and characterised; however, there are many limitations, such as poor stability which hindered the wide application of these enzymes. Thus, the search for better BVMOs has continued. This thesis reports the discovery of four BVMOs from the genome sequence of <i>Dietzia</i> sp. D5, a microorganism rich in oxygenases. The genes were cloned and expressed, and two of them were characterised. One of the enzymes, named BVMO3 readily oxidises linear aliphatic ketones. Characterization of the other BVMO, called BVMO4, revealed that it is the second most stable native BVMO ever reported and it oxidises a wide range of substrates. The oxidation of sulfides and aldehydes has been investigated further. Aldehydes were oxidised by this BVMO with a rare regioselectivity, producing carboxylic acid rather than formate ester which is the commonly observed product. Site saturation mutagenesis of selected amino acid residues in the proximity of the active site increased the cyclohexanone oxidation efficiency of BVMO4 by twelve-fold. | | |
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Baeyer-Villiger monooxygenases
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Enzyme discovery, characterization and engineering

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Abstract

With the emergence of Green Chemistry, biocatalysis is becoming an important approach in many laboratory and industrial processes. Enzymes catalyse chemical reactions with high regio- and stereoselectivity at mild conditions and, most importantly, are able to form products that are not possible to obtain by conventional synthetic chemistry.

Monooxygenases are a fascinating group of enzymes which oxidise substrates using atmospheric oxygen and release water as a by-product. These enzymes have great potential applications and indeed there are already some industrial showcases. Monooxygenases are of different types and one of the important groups is known as Baeyer-Villiger monooxygenases (BVMOs).

BVMOs oxidise ketones to esters and heteroatoms to the corresponding oxide, which are interesting reactions for pharmaceutical and fine chemical industry. To date, several BVMOs have been discovered and characterised; however, there are many limitations, such as poor stability which hindered the wide application of these enzymes. Thus, the search for better BVMOs has continued.

This thesis reports the discovery of four BVMOs from the genome sequence of *Dietzia* sp. D5, a microorganism rich in oxygenases. The genes were cloned and expressed, and two of them were characterised. One of the enzymes, named BVMO3 readily oxidises linear aliphatic ketones. Characterization of the other BVMO, called BVMO4, revealed that it is the second most stable native BVMO ever reported and it oxidises a wide range of substrates. The oxidation of sulfides and aldehydes has been investigated further. Aldehydes were oxidised by this BVMO with a rare regioselectivity, producing carboxylic acid rather than formate ester which is the commonly observed product. Site saturation mutagenesis of selected amino acid residues in the proximity of the active site increased the cyclohexanone oxidation efficiency of BVMO4 by twelve-fold.

Popular Summary

Most of the things that we use daily are the product of chemical reactions. Plastics derive from the polymerization of smaller molecules, drugs are synthesised rigorously by pharmaceutical industries, and products for the house and personal care contain a number of different chemicals and fragrances. All these compounds are produced by different branches of the chemical industry that rearranges raw material into more complex molecules.

Industrial chemical reactions can be subdivided into two categories: classical synthetic reactions and “green” biocatalysed reactions. Classic synthetic approaches often need high temperature and pressure to perform the reaction. Organic reactants are often toxic and generation and maintenance of high temperature and pressure consumes a great amount of energy. In the perspective of a world in which resources are limited and pollution is a problem for mankind, it is important to improve chemical reaction processes with the aim to reduce their energy requirements and to use non-toxic reactants. For this reason biocatalysed reactions are gaining more and more importance.

Biocatalysis as the name implies use biological catalysts, often referred as enzymes, to transform the raw material into product. Enzymes are produced by microorganisms including bacteria, yeast and fungi. Unlike chemical catalysts, enzymes are biodegradable, require mild conditions (low temperature and atmospheric pressure) to catalyse reactions, they are safe and specific. This thesis work aims at developing enzymes that can be used in the future to catalyse reactions.

Baeyer-Villiger monooxygenases are enzymes which insert one oxygen atom from atmospheric air into a substrate and release water as a by-product. The classical chemical process to catalyse the same reaction requires strong oxidants, that are toxic and carry a high risk of explosion and corrosion, to catalyse the same reaction. These enzymes can be potentially used to catalyse reactions for the production of drugs, perfumes and other chemicals for various applications such as in the production of biodegradable polymers, nylon, plastics etc.

This thesis focuses on Baeyer-Villiger monooxygenases (BVMOs) and in particular it deals with the discovery of new enzymes. Since existing BVMOs are rather unstable, meaning they lose the ability to catalyse the reaction in a short time, their application in industrial processes is hindered. Therefore there is a need for new and better industrial enzymes.

In this thesis work, four new enzymes are discovered from a bacterium called *Dietzia* sp. D5. One of these new BVMOs, is relatively stable compared to other enzymes of its kind and catalyses a number of reactions, including the synthesis of a precursor for

the anti-inflammatory drug family of profen (the drug marketed as Ipren in Sweden). This enzyme was also manipulated in order to produce more caprolactone, a precursor for the synthesis of different polymers. Mutation of the enzyme improved the production of caprolactone by 12-fold.

The work behind this thesis is just part of the global effort in moving towards a more sustainable chemical industry. It contributes by identifying a new source of oxygenase and novel BVMOs which can be potentially applied in the future to industrial processes.

List of papers

The thesis is based on the following papers, listed and referred in the thesis with roman numbers. The papers are attached as appendices at the end of the thesis. Reprints are reproduced with permission of the publishers.

- I. Serena Bisagni, Rajni Hatti-Kaul, Gashaw Mamo. “Insights into diversity of oxygenases in the draft genome sequence of *Dietzia* sp. D5 and analysis of the Baeyer-Villiger monooxygenases” (Manuscript submitted)
- II. Serena Bisagni, Justyna Smuś, Georgina Chávez, Rajni Hatti-Kaul, Gashaw Mamo. “Cloning and expression of a Baeyer-Villiger monooxygenase oxidising linear aliphatic ketones from *Dietzia* sp. D5” (Accepted ahead of print) *Journal of molecular catalysis B: enzymatic* doi: 10.1016/j.molcatb.2014.08.020
- III. Serena Bisagni, Rajni Hatti-Kaul, Gashaw Mamo. “Cloning, expression and characterization of a versatile Baeyer-Villiger monooxygenase from *Dietzia* sp. D5” (2014) *AMB Express* 4: 1-10.
- IV. Serena Bisagni, Benjamin Summers, Selin Kara, Rajni Hatti-Kaul, Gideon Grogan, Gashaw Mamo, Frank Hollmann. “Exploring the substrate specificity and enantioselectivity of a Baeyer–Villiger monooxygenase from *Dietzia* sp. D5: oxidation of sulfides and aldehydes” (2014) *Topics in Catalysis* 57: 366-375.
- V. Serena Bisagni, Milad Abolhalaj, Alexandre de Brevern, Joseph Rebehmed, Rajni Hatti-Kaul, Gashaw Mamo. “Enhancing the activity of a *Dietzia* sp. D5 Baeyer-villiger monooxygenase towards cyclohexanone by site saturation mutagenesis” (Manuscript in preparation)

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Paper III and **Paper IV** are reproduced by permission of Springer

My contribution to the papers

The initial idea came from Dr. Gashaw Mamo and Prof. Rajni Hatti-Kaul. Unless otherwise stated the work was carried on under their close supervision.

- I. I have analysed *Dietzia* draft genome sequence, analysed the Baeyer-Villiger monooxygenases and performed the experimental part. I have participated in the writing of the manuscript.
- II. I have performed part of the experimental work and supervised the work of Justyna Smuś and Gerogina Chavèz. I have written the first draft of the manuscript and coordinated the editing.
- III. I have planned and performed the experimental work and I have written the first draft of the manuscript.
- IV. The initial idea came from Dr. Frank Hollmann that supervised the work and helped in the writing of the manuscript. I have performed protein expression and the biocatalytic reactions. Help from Dr. Summers and Dr. Kara was received to setup the analytical protocols. Dr Gideon Grogan supervised part of the experimental work. I have coordinated the editing of the manuscript.
- V. I have performed part of the experimental work and supervised the work of Milad Abolhalaj. Dr. Alexandre de Brevern and Dr. Joseph Rebehmed have provided the homology model. I have helped in the editing of the manuscript.

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1. Introduction

In the last decades, the chemical industry has started to undergo major changes aiming at a more economical and environmental sustainability. The main driving force for the transformation of the chemical industry includes the depletion of the finite resources on our planet that the industry has been utilising and the increasing environmental conscious behaviour of human society.

A large variety of chemicals, materials and energy have been produced from fossil-based raw materials. These resources have been consumed for several decades that resulting in a reserve decline, which in turn drives the price of the raw materials upward. This is moving the switch towards using renewable resources such as crops, agricultural residues, forest biomass, etc. as alternative raw materials. Indeed, it is not only the price that gears the change but also the implementation of regulations at a national and international level to control and limit the use of toxic elements and volatile organic chemicals and generation of hazardous waste in chemical processes. These standards are summarised in the Principles of Green Chemistry, which give a guideline to industries on what is a green, sustainable process.

The principles are hereby listed according to Anastas and Warner [1] (Figure 1).

1. Prevent waste
2. Atom Economy
3. Less hazardous synthesis
4. Design benign chemicals
5. Benign solvents and auxiliaries
6. Design for energy efficiency
7. Use of renewable feedstock
8. Reduce derivatives
9. Catalysis (vs. Stoichiometric)
10. Design for degradation
11. Real-time analysis of pollution and prevention
12. Inherently benign chemistry for accident prevention

Figure 1: The twelve Principles of Green Chemistry according to Anastas and Warner

Chemical processes are multifaceted sets of operations that can be simply divided into three parts: the up-stream, the reaction phase and the down-stream part of the process. Specific principles of green chemistry apply to each of these parts, for example the preference of using renewable feedstocks over non-renewable (principle number 7) applies to the up-stream part and many other principles, such as number 1, 5 and 8 deal with the product recovery and waste handling of the process. The other principles instead underline that safe and energy efficient processes should be used (number 2, 3, 4, 6, 11 and 12) and in particular the principle number 9 proposes the use of catalysts rather than stoichiometric reagents. With respect to the latter principle, several catalysts i.e. heterogeneous catalysts and organocatalysts have been developed; the scope of this thesis however focuses on enzymes, the biological catalysts and their potential for implementing sustainable chemical processes.

Enzymes operate under mild conditions (usually at a temperature between 20 and 80 °C, mostly in aqueous solutions and atmospheric pressure) and hence require relatively low amount of energy to catalyse reactions. Today, enzymes constitute a well-established industry. Several companies around the world produce enzymes in bulk quantities with applications in very different fields. To name a few, they are included in detergents to improve the cleaning properties even at low temperatures, employed in food processing, added as supplements to animal feed to increase the quantity of bioavailable phosphorous, used at different steps in the process for paper production and recycling, and even employed in the production of biofuels.

Another enzyme market share that requires attention is the area of biocatalysis, where enzymes are used to catalyse chemical reactions. The use of enzymes for catalysing organic reactions has several advantages over the classical organic synthetic approaches, especially in terms of high regio- and enantiospecificity that makes it unnecessary to have complex protecting group, reducing the number of steps in the process, as well as the production of waste.

Although enzymes can be isolated from living organisms spanning the whole kingdoms of life, the majority of the enzymes used originate from different microorganisms including bacteria, yeasts, archaea and fungi. Especially interesting are the enzymes from extremophiles, microorganisms living in “unusual” extreme environments such as high temperature, high or low pH, high salt concentration, etc. Only about 1 % of the microorganisms have been grown and identified [2], suggesting the existence of an enormous catalytic pool still remaining to be discovered. Recent advances in bioinformatics, genomics, metagenomics, etc. has given access to a vast pool of genetic information that can be carefully analysed for choosing an optimal starting material to select a suitable biocatalyst for a reaction of interest.

It is now common practice to express the genes encoding enzymes in heterologous host. Generally, they are easy to cultivate and can produce higher amount of the

enzyme than the wild type microorganism. The enzymes may be further engineered by manipulation of the encoding gene using different techniques in order to improve activity, specificity and stability. Often, enzyme engineering is an iterative process and more cycles of mutagenesis are performed until the desired property is achieved. Further choices to be made include the form of biocatalyst (as free enzyme, immobilised preparation or whole-cells), reaction conditions, process format, etc.

At the current state, only a limited number of enzymes have been developed to the point of being used routinely to catalyse organic reactions in industrial processes. The enzyme group that has the biggest share of market is by far hydrolases (Enzyme Class 3) which are widely used in biosynthetic chemistry, and lipases are the best example [3]. Following hydrolases the class of enzymes that is rapidly increasing is oxidoreductases (EC 1) followed by transferases, lyases and ligases [3]. The reactions catalysed by oxidoreductases are several, to name a few they are capable of reduction of a carbonyl moiety to an alcohol, reduction of carbon-carbon double bond and insertion of an oxygen molecule at inactivated carbons. Oxidoreductases, as well as other enzymes, are especially interesting because they outclass their chemo-catalysed counterpart in reaching excellent selectivity of the reaction and they use cheap and readily available oxidant molecules, such as atmospheric oxygen in the case of oxidases and oxygenases.

1.1 Scope of the thesis

This thesis is focused on the enzyme development of novel Baeyer-Villiger monooxygenases (BVMOs).

BVMOs are enzymes that catalyse the oxidation of ketones to the corresponding ester. In addition, because of the promiscuity of these enzymes, they are also capable of oxidising aldehydes as well as heteroatoms, i.e. sulfides, nitrogen, phosphorous, selenium to the corresponding oxide [4]. The wide scope of reactions catalysed by BVMOs could find application in the synthesis of a wide variety of fine chemicals such as active pharmaceutical ingredients (API) as well as in applications in which bulk production is required, such as monomers for polyester production. However, their application in large-scale industrial processes has rarely been observed. Several reasons are limiting the use of BVMOs in synthetic applications, one of them is certainly the lack of stable and efficient enzymes. In this thesis the potential of BVMOs identified from a newly identified organism called *Dietzia* sp. D5 has been explored.

The thesis is composed of five manuscripts of which three are published or accepted.

Paper I deals with gene discovery, sequence analysis and initial screening of the biocatalytic properties of the four newly identified BVMOs. In addition, an effort was made to compare the number of oxygenase genes and sequence similarity among *Dietzia* strains and other oxygenase-rich microbial species.

Paper II and **Paper III** focus on the characterization of two of the BVMOs from *Dietzia* sp. D5. **Paper II** reports the heterologous production and characterization of BVMO3. Since it was not possible to purify this enzyme in active form, the substrate scope of the enzyme was determined using whole-cells and crude cell extract. In **Paper III**, the enzyme called BVMO4 was expressed and purified. The purified enzyme was characterised with respect to the optimal reaction parameters for activity, substrate scope and stability to different stresses.

Paper IV reports a more specific investigation of the substrate scope of BVMO4, focusing exclusively on two classes of substrates: aldehydes and sulfides. Sulfides have often been tested for sulfoxidation by BVMOs but no enzyme with similar sequence features to BVMO4 was ever tested with these substrates. The regioselectivity of BVMO4 for oxidation of aldehydes was found to differ from most of the other enzymes reported so far.

Paper V is based on engineering of BVMO4 to enhance its activity on conversion of cyclohexanone to caprolactone. Protein engineering was performed by site saturation mutagenesis after analysing the model structure of the enzyme and information retrieved from literature about mutations of other BVMOs.

The following chapters are intended to give an overview of the topics dealt with in the thesis and the papers, as well as some conclusions and future perspectives. Chapter 2 illustrates the progress of biocatalysis and the potential of oxidoreductases, especially monooxygenases. Oxidoreductases are a class of enzymes that has aroused a growing interest and that is slowly starting to become a workhorse for industrial biocatalysis after many years of academic investigation [5]. To this class of enzymes belong BVMOs, which are described in detail in Chapter 3. An extensive report about cloning, expression and characterization of the known BVMOs is provided and also the state of the art regarding mutagenesis and biotransformation with these enzymes is summarised in order to put in a wider perspective the results achieved in the five papers composing the thesis. Chapter 4 summarises the conclusion drawn from the studies as well as some reflections on the results obtained, future developments of this work and general limitations of BVMOs that should be considered in the perspective of developing better enzymes for industrial applications.

The work presented in the thesis was performed for the first three years within the Marie Curie Initial Training Network “Biotrains”. The network, involving universities and centres of excellence, and supported by industrial affiliated members, had the objective of training a group of young researchers (PhDs and PostDocs) “in

the applications of biocatalysis for sustainable chemical manufacturing”. This project was financed by the European Union through the 7th Framework People Programme, Grant Agreement no. 238531. The fourth year of PhD studies was supported by a grant from Swedish Research Council.

2. Oxidoreductases in biocatalysis

Oxidation-reduction reactions, also called redox reactions, are processes where electrons are transferred between two molecules. Many important processes in chemical industry, biology and geology are oxidation-reduction reactions. Processes such as combustion, corrosion, recovery of metals from ores, dissolving of metals, electrochemical processes in batteries and production of different kind of chemicals.

The process of life itself is driven by redox reactions. Cellular respiration and photosynthesis are examples of redox reactions fundamental for life. Without energy there is no life and biological energy is generated, stored and released by means of redox reactions. Photosynthesis involves the reduction of carbon dioxide into sugars and the oxidation of water into molecular oxygen. On the other hand, respiration oxidises the sugars to produce carbon dioxide and water. In addition, a vast array of metabolic reactions involve redox reactions and cells have evolved compounds such as NAD(P)H, FAD, ATP, Coenzyme A, etc. and a large number of enzymes known as oxidoreductases to perform the diverse and complex redox reactions.

Oxidoreductases are enzymes that catalyse reactions between two molecules in which one is oxidised and the other is consequently reduced. These enzymes are classified and grouped according to the Enzyme Commission as EC 1.

In industry, redox reactions are often catalysed using extreme conditions and catalysts that are unsafe to use and store. Moreover, reactions mediated by such conditions could be unspecific, generate toxic waste and side products. On the other hand, the use of oxidoreductases to catalyse redox reaction increases safety as these enzymes do not require high temperature and pressure, flammable solvents and strong oxidants [5]. In addition, oxidoreductases yield highly pure products since they are regio-, stereo-, or chemoselective [6]. Moreover, the use of enzymes as catalysts often reduces the amount or toxicity of waste. Hence, there has been a growing interest to use enzymes in redox reactions.

Over the years, different oxidoreductases have been developed and are being used at industrial scale. Pharmaceutical companies are among the industries where there is a sizable number of established processes that use oxidoreductases, such as in the production of chiral pharmaceutical intermediates [7]. For instance, the synthesis of a chiral building block for the synthesis of Lipitor[®], a drug used to control the blood level of cholesterol with annual sale of more than 10 billion dollars per year is one example. This synthesis uses a dehydrogenase, which reduces with high stereoselectivity the keto-group of ethyl 4-chloroacetoacetate [8]. Another example is monoamine oxidases which are used to produce a synthon of the anti-hepatitis drug telaprevir [9]. Other oxidoreductases, more precisely the combination of a

monooxygenase and dehydrogenases, have been used by Pfizer for the preparation of 2-quinolaxinecarboxylic acid [10]. This molecule is used in the synthesis of bioactive compounds and oxidoreductases are the catalyst of choice for this reaction because other approaches lead to the formation of toxic and thermosensitive intermediates. Moreover, biocatalytic oxidations have been used in the production of vitamin C [11], steroid hormones [12] and other intermediates for organic synthesis [13]. Although the current numbers of oxidoreductases used in industrial biocatalysis is limited, it is clear that their application in industrial oxidation reactions is expanding [14-15].

The advantages of using enzymes as biocatalysts has led to an increase in research, development and evaluation of different redox enzymes for various applications as observed in one of the most important conferences in the field [5]. Studies on well characterised hydrolases (e.g. lipases, esterases, proteases) have become less frequent, while research on oxidoreductases and transferases (mainly transaminases) are gaining momentum. Oxidoreductases and transaminases have been identified as interesting groups of enzymes for industry; however, as the application of enzymes at industrial scale requires robust and efficient biocatalysts, it is necessary to develop enzymes with better stability and activity [5, 16-17].

Based on the type of reactions they catalyse oxidoreductases are classified by the Enzyme Commission in 22 sub-classes. These enzymes were broadly categorised into four groups - dehydrogenases/reductases, peroxidases, oxidases and oxygenases/hydroxylases [18]. However, for simplicity, in this thesis oxidoreductases are divided into two major groups - enzyme which require oxygen and those which do not (Figure 2).

2.1. Oxidoreductases which do not require oxygen

This group includes enzymes that oxidise substrates without involving oxygen. Few examples of these enzymes that are important in biocatalytic applications are shown in Figure 2 and a brief description is given below.

Dehydrogenases include all those enzymes that catalyse the oxidation of a substrate upon reduction of another and involve the transfer of a hydride. In particular, alcohol dehydrogenases (ADH, EC 1.1.1.1), catalyse the reduction of a carbonyl group (a ketone or an aldehyde) into a primary or secondary alcohol (Figure 3). In association to this reaction, the cofactor NAD(P)H (nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate) is oxidised to NAD(P)⁺. Interestingly this reaction can be reversed: the alcohol is used as a substrate, and ketone is produced together with reduced NAD(P)H (Figure 3). The regeneration of NAD(P)H is an

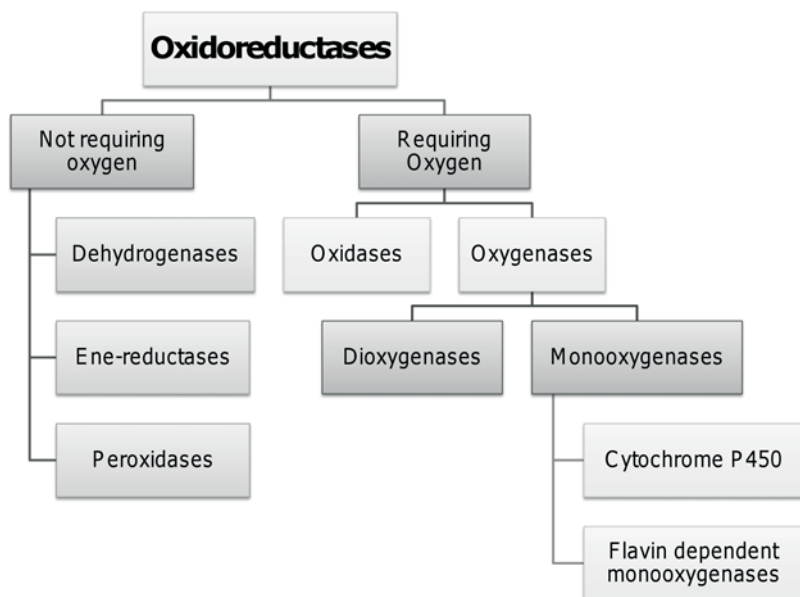


Figure 2: Classification of oxidoreductases in two major groups: enzymes that require oxygen and those which do not require oxygen

important issue in many biocatalytic reactions where these cofactors are involved. The cofactors are expensive and cannot be used in stoichiometric amount to the substrate and hence must be regenerated. Therefore cheap alcohol substrates (e.g. ethanol, isopropanol) can be oxidised by an ADH to produce NAD(P)H that will be taken up by another NAD(P)H dependent enzyme, such as monoxygenases. In addition to their application in cofactor regeneration systems, ADHs are becoming important tools in organic synthesis [5, 19].

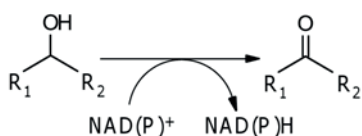
Ene-reductases reduce α - β unsaturated bonds which is a reaction that can generate up to two chiral centers and it has a great potential for the production of stereo-pure products (Figure 3) [20]. The best studied ene-reductase is the Old Yellow Enzyme (OYE) and it is a flavin mononucleotide (FMN)-containing enzyme which uses NAD(P)H as reducing agent [20]. OYE has been reported to react with α - β insaturated ketones, aldehydes, carboxylic acids and nitroalkenes [21]. These compounds are of high interest for pharmaceutical applications as well for the flavor industry such as for the production of enantiopure menthol [22].

Peroxidases are a diverse group of enzymes with different cofactor requirements. The most common peroxidases are heme enzymes in which the iron center generates radical species using hydrogen peroxide as electron acceptor [6]. Peroxidases are versatile enzymes catalysing a number of reactions, such as the oxidative dehydrogenation of phenols and catechols, heteroatoms oxidation and epoxidation (Figure 3) [6]. The most commonly used peroxidases in organic synthesis are

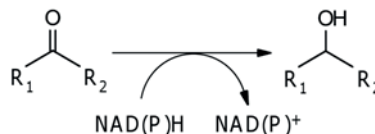
chloroperoxidase and horse radish peroxidase. Other peroxidases with different cofactors are metal dependent peroxidases containing manganese, vanadium or selenium. They have a great industrial potential in the detoxification of wastewater, dye bleaching and in clinical devices [23]. These enzymes could be of great interest for the production of polymeric materials by radical condensation [24] and their implementation for this purpose is currently under investigation.

Dehydrogenases

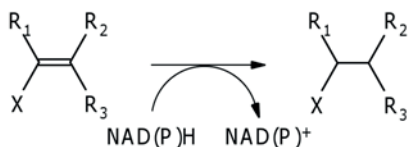
Oxidative reaction



Reductive reaction

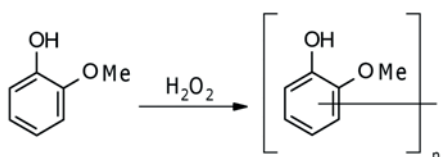


Ene-reductases

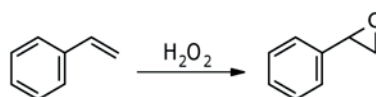


Peroxidases

Oxidative dehydrogenation of phenols



Epoxidation



Heteroatom oxidation, i.e. sulfoxidation

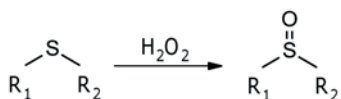


Figure 3: Representative reactions for dehydrogenases, ene-reductases and peroxidases. R₁, R₂ and R₃ are generic substituents; X represents an electron withdrawing group.

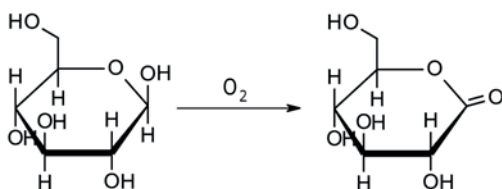
2.2. Oxidoreductases which require oxygen

According to the proposed subdivision of oxidoreductases, those that require oxygen include oxygenases and oxidases, which differ in the way oxygen is used during the catalysis. Oxidases use molecular oxygen as the electron acceptor which is released as hydrogen peroxide or water, whereas oxygenases incorporate one or both atoms of the oxygen molecule into the substrate. The fascinating feature of this group of enzymes is the ability to activate the kinetically inert oxygen molecule into highly oxidising species.

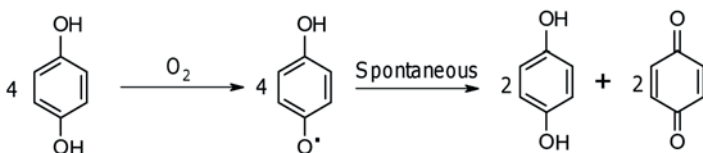
Oxidases are a very diverse family of enzymes that catalyse the oxidation of carbon-oxygen and carbon-nitrogen bonds. The most common cofactors for oxidases are either copper or flavin [25]. Glucose-2-oxidase, laccase and monoamine oxidase can be mentioned as examples of oxidases which have found relevant industrial applications (Figure 4).

Oxidases

Glucose-2-oxidase



Laccase



Monoamine oxidase

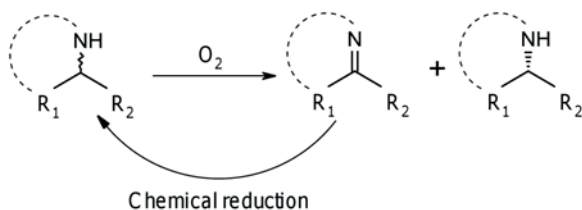


Figure 4: Reactions catalysed by different oxidases. R₁ and R₂ are generic substituents, Monoamine oxidase can react with primary, secondary and tertiary amines.

Glucose-2-oxidase, that oxidises glucose to gluconolactone (Figure 4). It is applied in the food industry and it is also used in devices monitoring glucose level, such as test-kits for monitoring blood sugar level in diabetic patients. Laccases catalyse the oxidation of phenols (Figure 4), polyamines and lignin. They have a number of applications in the textile, pulp and paper industry, and bioremediation [5, 26] as well as in synthetic reactions employing dyes and polymeric materials [27-28]. Monoamine oxidases reduce amines to imines. These enzymes are very selective and hence can produce chiral amines by kinetic resolution of the achiral amine mixture (Figure 4). Chiral amines are highly regarded for the production of pharmaceutical intermediates that can also be obtained using transaminases [29].

Oxygenases catalyse the insertion of oxygen atoms in organic substrates. Oxygenases are known to catalyse complex oxidations such as oxidative cleavage of carbon-carbon bonds, monohydroxylation and dihydroxylation reactions. Insertion of oxygen is a difficult reaction to achieve by classical synthetic approaches, which generally require strong oxidising agents that usually have poor regio- and stereoselectivity. Instead, oxygenases overcome this limitation by producing highly oxidising species using molecular oxygen that is activated in the active site of the molecule. To do so, most of the oxygenases have a cofactor, such as flavin, heme or metal ions. To keep the redox cycle in balance, it is usually necessary to regenerate the cofactor back to its initial redox state. However, some cofactor independent oxygenases are also known [30].

Oxygenases are divided into two groups based on the number of oxygen atoms that the enzyme inserts into the substrate. If both the oxygen atoms are incorporated into the substrate, the enzyme is referred to as dioxygenase, while a monooxygenase catalyses the insertion of only one oxygen atom in the molecule and the other one is reduced to form water.

2.2.1 Dioxygenases

Most dioxygenases have iron-containing cofactors (heme or coordinated iron) [31]. Some flavin dioxygenases and cofactor independent dioxygenases have also been reported [32-33]. Dioxygenases are known for their broad substrate specificity, which makes these enzymes one of the attractive biocatalysts for redox reactions.

One good example is Toluene dioxygenase of *Pseudomonas putida*, which catalyses the oxidation of over 100 different substrates [34]. This enzyme can transform arenes and alkenes to *cis*-dihydrodiols. Moreover, it hydroxylates saturated carbon atoms and oxidises sulfides to chiral sulfoxides [35]. Toluene dioxygenases is part of the wider group of aromatic dioxygenases. As the name suggests these enzymes oxidise aromatic molecules and catalyse the *cis*-hydroxylation of aromatic rings. If a substituent (i.e. chlorine, nitrate or sulfite) is present on the aromatic ring, the dioxygenases can catalyse simultaneously the *cis*-hydroxylation and also the elimination of such group. Toluene dioxygenase from *P. putida* has also been used for the synthesis of

pharmaceutical precursor for the synthesis of the pharmaceutical Indinivir (Figure 5), a protease inhibitor used in formulation of anti-HIV drug [34].

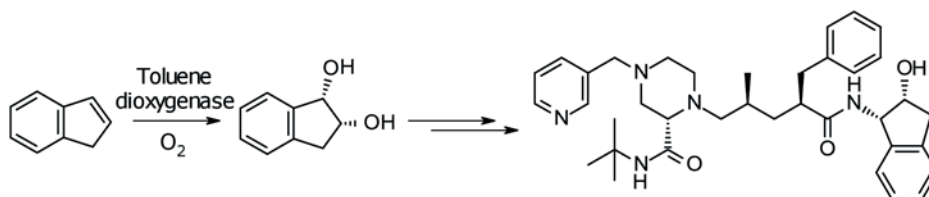


Figure 5: The reaction catalysed by Toluene dioxygenases for the synthesis of a precursor of Indinivir.

Another example of interesting dioxygenases are lipoxygenases. They are enzymes that act on polyunsaturated alkenes and catalyse the dihydroxylation of such molecules to their hydroperoxy form (Figure 6). This reaction is interesting for producing, for example, hydroxyacids by reduction of the hydroperoxy group. Hydroxyacids can be used in a number of applications like in production of resins, waxes, bioplastics, etc. In addition, lipoxygenases *in vivo* are involved in the production of signalling molecules (i.e. γ -decalactone and γ -dodecalactone) that can be used as pharmaceuticals or in the flavour industry [36].

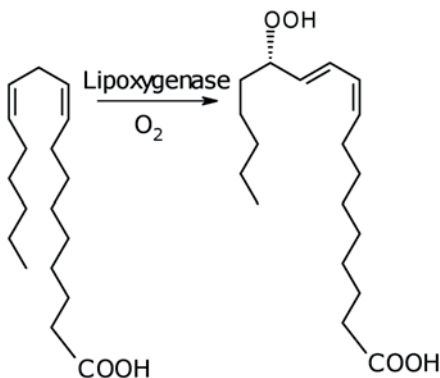


Figure 6: Peroxygenation of linolenic acid by a lipoxygenases.

However, despite their remarkable catalytic properties, dioxygenases are not widely used in industry. One of the reasons could be that these enzymes are formed by independent sub-units which make it difficult to obtain good enzyme activity in cell-free system and this restricts their use only as whole-cell systems. Today, most of the enzymes used in large scale applications are recombinantly expressed; however, heterologous expression of dioxygenases is difficult which further hinders their application.

There is ongoing research, although at a low pace, that makes the future of dioxygenases in biotransformation applications bright. For instance, the biotransformation of phenolic ring to chiral *cis*-cyclohexenone diols has been explored in detail [37] and this discovery can lead to the possibility of using dioxygenases to transform phenols from renewable resources into useful products.

2.2.2 *Monoxygenases*

As mentioned above, monooxygenases catalyse the insertion of one oxygen atom into a substrate. To perform this catalysis, the enzyme requires a cofactor that activates the otherwise inert oxygen to make it available for the reaction. The cofactors that are most frequently encountered in monooxygenases are flavin cofactors (FMN or FAD) and iron centres, either as a single iron atom coordinated with a porphyrin or as multiple iron centres coordinated to residues in the protein. Although not abundant, copper and pterin-dependent monooxygenases are also reported [38].

In addition, a limited number of monooxygenases can also activate the dioxygen without cofactor. Although the elucidation of the mechanism of this enzyme is still ongoing, it has been indicated that the oxygen activation occurs in the active site starting from the generation of a carbanion in the substrate that leads to the generation of the peroxyanion-intermediate [39]. The cofactor independence of these enzymes makes them an appealing option for biocatalytic applications, however their substrate scope is very limited and it cannot be easily improved as the substrate has to form and stabilise a carbanion [38].

2.2.2.1 **Heme-dependent monooxygenases**

Heme-dependent monooxygenases are also called cytochrome P450 or CYPs. They contain a prosthetic group constituted of a heme b, in which the iron atom coordinates the four nitrogens from the porphyrin (Figure 7). The fifth coordination position is occupied by a cysteine that is part of the protein sequence of the cytochrome P450. The cysteine activates the iron centre and is conserved in all cytochrome P450s [40]. CYPs require NAD(P)H to complete the oxidation reaction and since a direct electron transfer from the NAD(P)H to the porphyrin is not possible, another enzyme for the reduction is needed. It is constituted of a ferredoxin and a ferredoxin reductase. CYPs can be further divided into four classes depending on if the three subunits are fused into a single peptide or not and also if the enzymes are soluble or membrane bound [38].

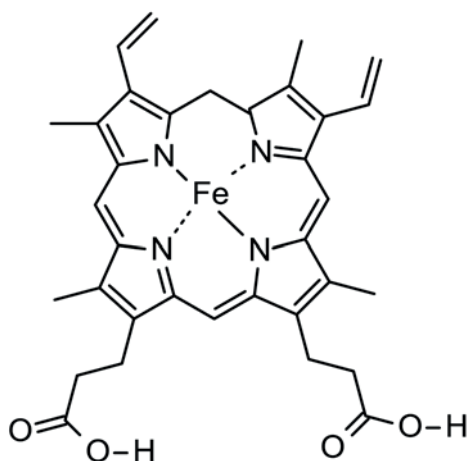
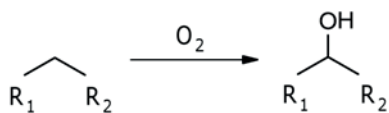


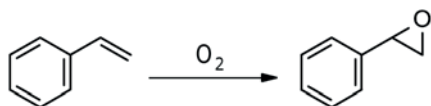
Figure 7: Heme b coordinating an iron atom. In the resting state the iron is in oxidation state II.

Cytochrome P450 monooxygenases

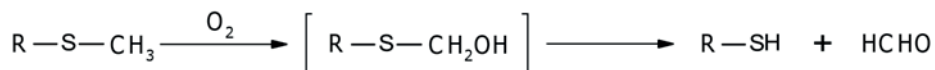
Hydroxylation



Epoxidation, heteroatoms-dealkylation and oxidations



Heteroatom-dealkylation (i.e. sulphur)



Heteroatom oxidation (i.e. sulphur)

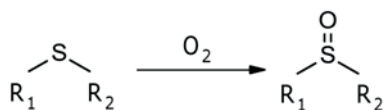


Figure 8: Examples of reactions catalysed by cytochrome P450. R, R₁ and R₂ are generic substituents.

Cytochrome P450s can be found in eukaryotic and prokaryotic organisms in highly varying numbers. For example, *Orzya sativa* contains 455 genes encoding for CYPs [41], humans have 57 different genes [42], *Streptomyces avermitilis* encodes for 33 cytochrome P450s [43] and *E. coli* has no CYP encoding genes [40].

These enzymes can catalyse different reactions such as hydroxylation, epoxidation, heteroatoms-dealkylation and oxidation (Figure 8) [44]. Thus, there is a great interest in using cytochrome P450s in different applications. However, despite their great potential, currently CYPs are sparingly employed in industry due to their low stability [38].

One application of CYPs at the current development state is the production of drug metabolites that would be produced by the detoxification machinery in the human body [45]. These metabolites are used in toxicological studies by pharmaceutical industries and their synthesis is hard to achieve by other chemical routes. Hydroxylation at non-activated carbons is a very attractive reactivity of CYPs. In fact, this reaction is hard to perform by chemical based synthesis because of overoxidation of the substrate. However CYPs prove to be a better alternative since they are selective and do not overoxidise the substrate. Similarly, preparation of active pharmaceutical intermediates by CYPs is attractive and especially the hydroxylation of steroids is of great interest. For instance, the production of cortisol starting from 11-deoxycortisone was demonstrated at lab scale and it was possible to produce 1 mM of this product after 72 hours of reaction [46].

It seems evident that the use of CYPs needs to be perfected and several problems have to be solved. For instance, CYPs need for their functioning a reductase system but the co-expression of three genes could be cumbersome. Preparation of polycistronic plasmids or fusion of the sub-units into one polypeptide has been tried [40]. In addition to the reductase, a system for the regeneration of NADPH has to be provided. Both enzymatic and non-enzymatic cofactor recycling methods such as the electrochemical reduction of the heme in CYPs have been reported [47]. Moreover, wild type enzymes can have low activities with substrates of industrial interest, therefore protein engineering is a necessary step to make the biocatalyst more suitable for the task [48]. Finally, substrates for CYPs are usually hydrophobic molecules with low solubility in water systems. Engineering of the reaction as well as enzyme engineering have been investigated for CYPs catalysed reactions [49].

Overcoming the above mentioned limitations would pave the way for synthesis of an enormous number of products using CYP mediated reactions.

2.2.2.2 Flavin dependent monooxygenases

The family of flavin dependent monooxygenases is a very diverse group of enzymes whose common characteristic is to have a flavin cofactor that allows oxygen insertion to a substrate. The cofactor itself can differ, being either flavin adenine dinucleotide

(FAD) or flavin mononucleotide (FMN) (Figure 9). On the basis of the type of cofactor, the protein fold, the oligomeric state and the type of reaction catalysed, flavin dependent monooxygenases can be divided into 8 groups (Table 1) [50].

Table 1: Classification of flavin dependent monooxygenases according to Huijbers and co-workers [50].

| Group | Cofactor | Electron donor | Protein fold | Reaction |
|-------|-------------|--|--------------------------------|---|
| A | FAD | NAD(P)H | Rossmann (GR-2) | Hydroxylation, sulfoxidation |
| B | FAD | NAD(P)H | Rossmann (FMO) | Baeyer-Villiger oxidation, heteroatom oxydation, N-hydroxylation, oxidative decarboxylation |
| C | FMN | FMNH ₂ | Tim-barrel (luciferase) | Light emission, Baeyer-Villiger oxidation, epoxidation, desulfurization, sulfoxidation, hydroxylation |
| D | FAD/ FMN | FADH ₂ / FMNH ₂ | Acyl-CoA dehydrogenase | Hydroxylation, N-hydroxylation |
| E | FAD | FADH ₂ | Rossmann (GR-2) | Epoxidation |
| F | FAD | FADH ₂ | Rossmann (GR-2) | Halogenation |
| G | FAD | Substrate | Rossmann (MAO) | Oxidative decarboxylation |
| H | FMN | Substrate | Tim-barrel (glycolate oxidase) | Oxidative decarboxylation |

Flavin is fundamental for catalysis as it binds oxygen to form the peroxide intermediate, called C4a-hydroperoxyflavin that reacts with the substrate generating the hydroxylated product. In order for the flavin to bind oxygen it has to be in the reduced state and the reducing equivalents are generally obtained by other reduced coenzymes, such as NAD(P)H, FMNH₂ or FADH₂ (Figure 9).

The spectrum of reaction catalysed by flavin dependent monooxygenases as a whole is very diverse and accounts for *ortho*- and *para*-hydroxylation of phenols, Baeyer-Villiger oxidation, epoxidation, heteroatom oxidation and oxidation of aldehydes (Figure 10) [51]. The hydroxylation of phenols is a reaction that can be hardly be performed by synthetic chemistry. Therefore the investigation of flavin dependent hydroxylases is currently ongoing to provide efficient enzymes for this task [52].

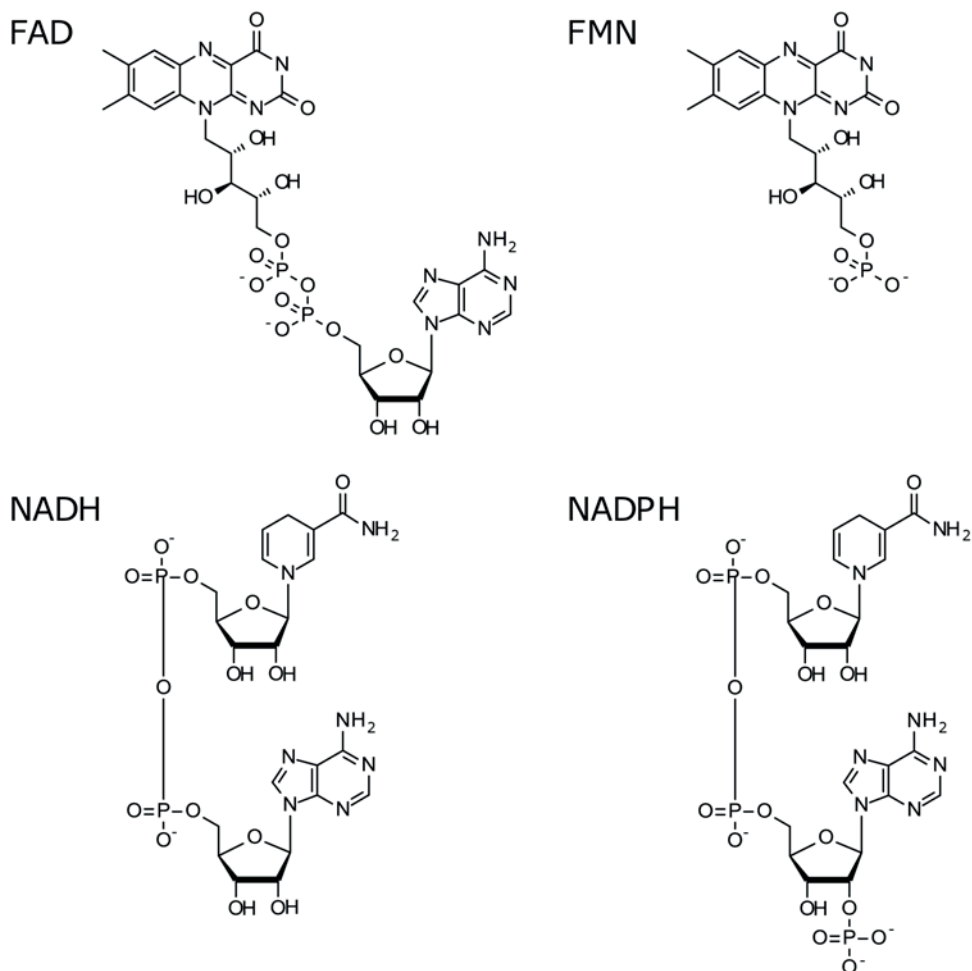


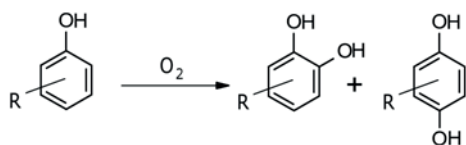
Figure 9: Flavin and nicotinamide cofactors used in flavin dependent monooxygenases.

Flavin monooxygenases (FMOs) and Baeyer-Villiger monooxygenases (BVMOs) are of special interest in this thesis. Both enzymes have been classified as class B [50, 53]. The enzymes of this class are formed by a single peptide chain which has two dinucleotide binding domains (called Rossmann folds) that bind respectively FAD and NAD(P)H. Non covalent interactions tightly secure FAD inside the enzyme, while NAD(P)H enters and leaves the enzyme during the catalytic cycle. NADPH is usually the preferred cofactor but some FMOs can also use NADH [54-55].

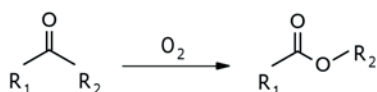
Internal flavin dependent monooxygenases are an exception to the above mentioned requirement for a reduced coenzyme and few examples have been reported. Internal flavin monooxygenases work similarly to internal CYPs as the flavin of these enzymes is reduced using electrons coming from the substrate and therefore does not require NAD(P)H, FMNH₂ or FADH₂ for reducing the flavin [56-57].

Flavin-dependent monooxygenases

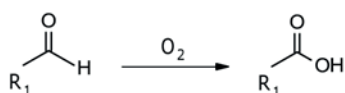
ortho- and *para*-hydroxylation of phenols



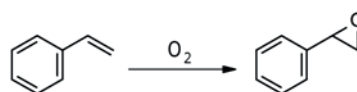
Baeyer-Villiger oxidation



Oxidation of aldehydes



Epoxydation



Heteroatom oxidation (i.e. sulphur)

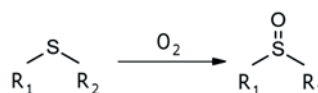


Figure 10: Representative reactions catalysed by flavin dependent monooxygenases. R, R₁ and R₂ are generic substituents.

As stated above, FMOs and BVMOs in part have similar folding but the FMOs amino acid (aa) sequence length can vary between 350 aa to more than 600 aa, while BVMOs are usually between 500 and 650 aa long. FMOs and BVMOs catalyse oxidation of heteroatoms and Baeyer-Villiger reaction. However, FMOs have a quite narrow substrate scope for Baeyer-Villiger oxidation [54-55].

Unlike BVMOs, FMOs are found in all the kingdoms of life. In humans, different isoforms of FMOs are found in the liver and they detoxify hydrophobic compounds by hydroxylating them, favouring their elimination. Their activity complements that of cytochrome P450s which are also found in the liver and have the same physiological role. However, CYPs are an established topic of study in biocatalysis, both from academia and industry, while FMOs are just starting to attract researchers' attention [54-55, 58-59].

BVMOs are in general categorised into three types. Type I BVMOs, the most studied group among the three types, are Class B flavin dependent monooxygenases which contain FAD and require NADPH for flavin regeneration. Type II BVMOs are Class C flavin dependent monooxygenases which are constituted by a FMN containing subunit and a reductase subunit that binds NADH [60]. Some examples of Type II BVMOs have been reported [61-64]. The third group is Type O BVMOs which are the least known BVMOs. They can be classified as Class A flavin dependent

monoxygenases, bind FAD and require NAD(P)H for reducing the flavin [65]. The folding and the structure of Type O BVMOs differ from those of Type I BVMOs, although some similarity remains regarding the FAD binding and the presence in both enzymes of some residues necessary for catalysis such as the “catalytic arginine” [65]. The only Type O BVMOs investigated to date are those involved in the secondary metabolites production in *Streptomyces* spp. [66-67].

This thesis focuses on Type I BVMOs and unless specified, the term BVMOs hereafter will be used to refer to Type I BVMOs.

3. Baeyer-Villiger monoxygenases

Baeyer-Villiger monoxygenases (BVMOs) are flavin monoxygenases that catalyse the Baeyer-Villiger reaction, oxidation of ketones to esters [68]. In the chemical based reaction, the ketone reacts with strong oxidants such as *m*-chloroperoxybenzoic acid, peroxyacetic acid or peroxytrifluoroacetic acid, to form the ester. However, the oxidant compounds are intrinsically unstable. They are used in stoichiometric amounts meaning that large volume of peroxyacids are handled, stored and transported, implying the risk of corrosion and explosion. For this reason, enzymatic alternatives for the Baeyer-Villiger reaction are preferred over the chemical catalysed reaction.

BVMOs use atmospheric oxygen as oxidant instead of the peroxyacids, therefore avoiding the safety limitations of the classic Baeyer-Villiger reaction (Figure 11). The first BVMOs were discovered almost forty years ago by Trudgill and co-workers [69-71] and since then a large number of BVMOs have been reported.

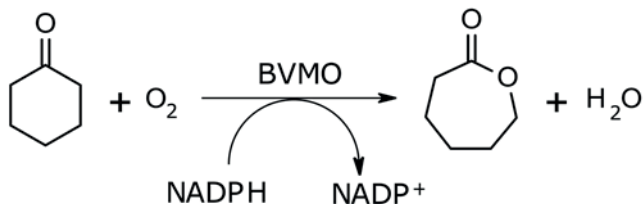


Figure 11: Baeyer-Villiger oxidation of cyclohexanone yielding caprolactone catalysed by a generic BVMO.

The proposed reaction mechanism proceeds through several steps and starts with the reduction of FAD by NADPH. The reduced flavin allows the binding of the oxygen and the formation of the peroxy-adduct with FAD. The newly formed highly reactive oxygen species can then react with the ketone forming the Criegee intermediate that spontaneously rearranges, yielding the ester product. In the meanwhile, the other oxygen atom is released from the FAD as a water molecule. The oxidised cofactor NADP⁺ formed in the first step remains bound to the enzyme active site throughout the whole reaction cycle although it has no active role in the reaction anymore. The presence of NADP⁺ in the active site is proposed to have a role in stabilising the protein-substrate structure throughout the reaction [72]. Finally, the reaction terminates when NADP⁺ leaves the active site (Figure 12).

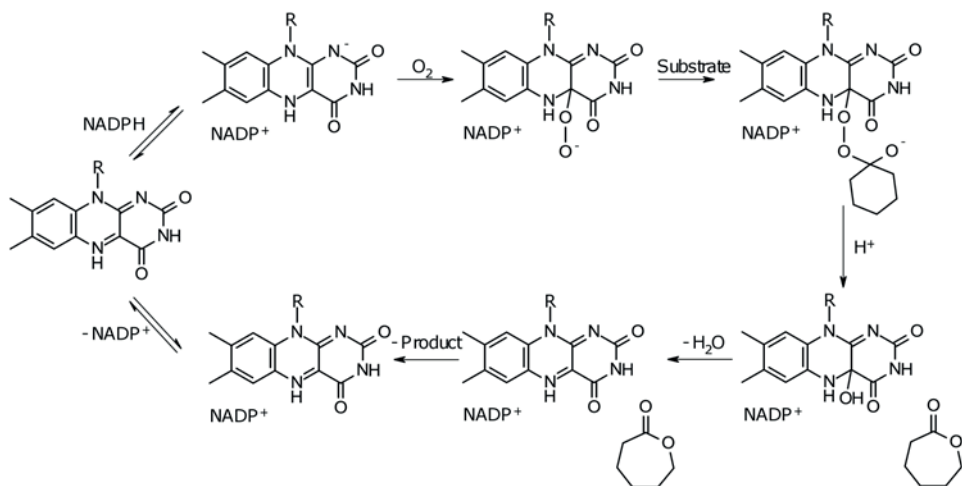


Figure 12: Reaction mechanism of BVMO as adapted from [73].

3.1 Sources of BVMOs

BVMOs have been found in prokaryotic organisms as well as in eukarya and archaea. The physiological role of BVMOs in an organism varies depending on its involvement in primary or secondary metabolism. In primary metabolism, BVMOs have been shown to participate in the oxidation of hydrocarbons, which are finally used as carbon and energy sources by the organism [69, 74-75]. For instance, the oxidation pathway contains usually an alcohol dehydrogenase for the oxidation of a secondary alcohol to a ketone, then the BVMO oxidises the ketone to the ester and finally an esterase hydrolyses it to ω -hydroxyacid, which is further oxidised and enters the β -oxidation pathway where it will be further transformed to intermediates of the Krebs cycle (Figure 13).

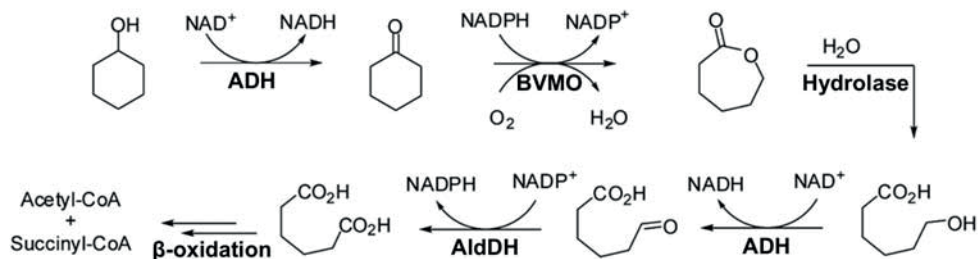


Figure 13: Proposed metabolic pathway for the oxidation of cyclohexanol. ADH stands for Alcohol dehydrogenase and AldDH is aldehyde dehydrogenase (adapted from [76]).

Some BVMOs are involved in the production of secondary metabolites such as pentalenolactone an antibacterial, antiviral and anticancer compounds, and insecticides belonging to the loline alkaloid family [77-78].

Earlier, BVMOs were discovered directly from wild type organisms [69-71]. Today, BVMOs are identified from microorganisms through screening processes, analysis of genomes and metagenome sequences. The understanding of BVMOs role has been improving with the advent of bioinformatics, genomics and molecular biology that has allowed researchers to analyse and compare genomes and find suitable enzymes and their sources [74, 79]. The increasing amount of knowledge from genome sequencing has revealed an enormous amount of information about several microorganisms and therefore genome mining has become a very efficient strategy for the discovery of new BVMOs, as well as other enzymes. One of the most studied BVMOs, Phenylacetone monooxygenase (PAMO) from the thermophilic organism *Thermobifida fusca* was discovered by genome mining [80]. Similarly, from the genome of *Rhodococcus jostii* 23 BVMOs were identified, and 6 BVMOs were also found in the genome of *Mycobacterium tuberculosis* [81-82].

From the enrichment culture of an activated sludge of a wastewater treatment plant mRNA was recovered and used as a template to amplify protein encoding gene sequences. Sequencing of the amplified fragments resulted in three new BVMOs and other genes related to the catabolism of hydrocarbons [83]. In a similar approach, from the genomic DNA of an organism whose genome is not sequenced, BVMOs could be obtained using the primer walking technique [84-86]. Especially in the case of eukaryotic organisms, primer walking might be advantageous because of the much larger size of the eukaryotic organisms genome than that of bacteria which makes the genome sequencing expensive and more challenging [87].

BVMOs have been identified from a number of organisms [76], although, to my knowledge, there are no BVMOs reported from higher organisms. In the last few years, BVMOs from a limited number of fungi and algae were recombinantly expressed and studied [87-90]. However, Baeyer-Villiger oxidation in fungi has been known for over twenty years [91-92].

Bacteria have been the most important sources of BVMOs and have been widely investigated. BVMO encoding genes have been reported in both gram-positive and gram-negative bacteria. Members of classes Gammaproteobacteria (*Acinetobacter* spp., *Pseudomonas* spp.), Betaproteobacteria (*Brachymonas* spp., *Comamonas* spp.), Alphaproteobacteria (*Xanthobacter* spp.) and Actinobacteria (*Arthrobacter* spp., *Brevibacterium* spp., *Gordonia* spp., *Mycobacterium* spp., *Nocardia* spp., *Rhodococcus* spp., *Streptomyces* spp.) are known to produce one or more BVMOs.

Some organisms that belong to Actinobacteria like *Rhodococcus jostii* RHA1 and *Mycobacterium tuberculosis* H37Rv are known to harbor multiple BVMO genes as

noted above [81, 93]. Similarly, *Dietzia* sp. D5, the organism that has been the source of the BVMOs studied in this thesis, belongs to the class of Actinobacteria and four putative BVMO genes have been identified in its draft genome sequence (**Paper I**).

3.1.2 *Dietzia* sp. D5 - a new source of novel BVMOs and other oxygenases

The growing interest in using BVMOs in different applications has been the impetus for searching novel and better enzymes. This thesis takes part in this global effort of looking for new BVMOs. The genus *Dietzia* has been described fairly recently and the first *Dietzia* strain, *Dietzia maris* was previously classified as *Rhodococcus* [94].

Dietzia sp. D5 is a Gram positive, mesophilic and alkaliphilic bacterium with high GC content in the DNA. *Dietzia* sp. D5 cells are rod-shaped and form bright orange colored colonies. This organism was isolated from a soil sample of Lake Abijatta, a soda lake in the Ethiopian Great East African Rift Valley. The soda lake isolates were screened for BVMO production by growing the cells in minimal medium containing cyclohexanone as carbon source. The organisms that grew in such medium were lysed and the crude extract was tested for BVMO activity. Three isolates showed good activity and the one that outperformed the others was “isolate D5” which was later identified as a strain of *Dietzia* and referred to as *Dietzia* sp. D5.

In **Paper I**, four new BVMO encoding genes from the draft genome sequence of *Dietzia* sp. D5 were identified. This organism is closely related to *Gordonia*, *Nocardia*, *Rhodococcus* and *Mycobacterium*, which belong to the suborder Corynebacterineae and that often contained one or more genes of BVMOs.

In addition to BVMOs, the genomes of these microorganisms are also very rich in other monooxygenases and dioxygenases. For example, *M. tuberculosis* H37Rv encodes for 41 monooxygenases and 11 dioxygenases while *R. jostii* RHA1 encodes for 129 monooxygenases and 85 dioxygenases [95-96]. It has to be considered that the length of *R. jostii* genome is twice the size of the *M. tuberculosis* genome. The analysis of the genome of *Dietzia* sp. D5 revealed the presence of 28 monooxygenases and 15 dioxygenases.

Besides the genome of *Dietzia* sp. D5, genomes of three other *Dietzia* spp. have been sequenced and deposited in databases until June 2014. At the time of writing **Paper I**, there were only two genomes available in the database. The growing number of genome sequences might indicate the increasing interest in these organisms.

The genome size of *Dietzia* spp. is in the range of 3.3 to 3.9 megabases and its GC content is high, 67 to 71%. The comparison of the oxygenases content in these *Dietzia* spp. genomes shows that the number of such genes per genome is somewhat constant throughout the strains (Figure 14). In general, *Dietzia* spp. have similar

relative oxygenase content as the known oxygenase rich organism, *M. tuberculosis*. The identification of several oxygenases from *Dietzia* spp. (including *Dietzia* sp. D5) suggests that this group of organisms potentially will serve as sources of novel oxygenases for the oxidation of a wide range of substrates.

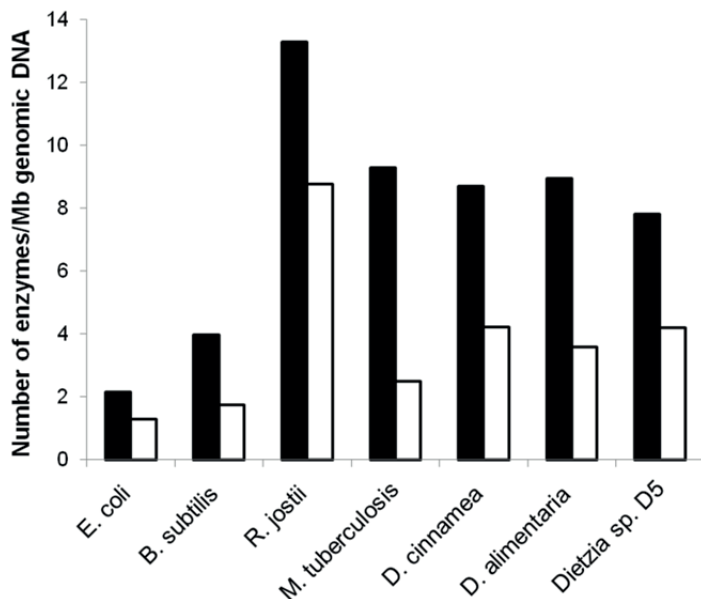


Figure 14: Comparison of the number of oxygenases per megabase of genomic DNA among *Dietzia* spp. and the oxygen rich organisms *R. jostii* RHA1 and *M. tuberculosis* H37Rv. *E. coli* and *B. subtilis* were used as negative controls. The black bars represent the monooxygenases and the white bars are for the dioxygenases.

Dietzia spp. are proven to have the ability to metabolise hydrocarbons and some of them were even isolated from petroleum contaminated environmental samples [97-98]. Studies have shown that certain strains can utilise hydrocarbons as the only carbon source [99] and others were proven to be able to bioremediate oil spills [100-104], hence suggesting the presence of oxidative enzymes used in hydrocarbon degradation pathways. *Dietzia* spp. have been considered for different applications in the field of veterinary medicine [105], production of carotenoids [106], biosurfactant [107-108] and biodemulsifier [109]. Although *Dietzia* spp. can serve as sources of interesting enzymes for biocatalytic applications [110], there is no significant effort made to tap their potential. So far, there is only one cytochrome P450 from *Dietzia* sp. DQ12-45-1b that has been recombinantly expressed in *E. coli*. One of the obstacles that hindered the wide expression and evaluation of enzymes from *Dietzia* spp. could be their high GC content and the relatively frequent rare codons which make expression in the common *E. coli* systems difficult. The studies reported in

Paper I, Paper II and Paper III contribute towards offering an insight on challenges of cloning and expression genes from *Dietzia* spp.

3.2 Primary structure analysis of BVMOs

Analysis of several BVMO sequences through comparative alignment studies led to the identification of special sequence features that characterise this type of enzymes.

Motifs shared by all the BVMOs are:

- **Two Rossmann motifs**: Rossmann motifs are short conserved glycine fingerprints (Table 2) that binds an ADP moiety, such as the one constituting NAD(P)H or FAD. In BVMOs, each of these motifs are responsible for the binding of one of the cofactors, FAD and NADPH [111].
- A **BVMO motif**: this sequence is typical for type I BVMOs which bridges the FAD and NADPH binding domains. The BVMO motif contains a highly conserved histidine. Although this region is not directly involved in catalysis, it plays a critical role in the enzyme conformation [111-112].
- A recently identified **BVMO motif**: these residues are close to the N-terminal part of the protein and are expected to play an important role during the catalytic cycle since the aspartic acid residue is highly conserved and has an important role in interaction with NADP⁺ [93].

In addition to characterising motifs, there are also amino acid residues that are highly conserved and have a crucial role for the folding and activity of BVMOs. They include:

- The **catalytic arginine**: a residue that is fundamental for catalysis. This residue seems to mediate the interaction between the nicotinamide of NADPH, the flavin and the substrate during the catalytic cycle [72]. Mutation of this residue to alanine resulted in a completely inactive enzyme, showing the importance of this residue [113].
- The **NADPH stabilising arginine**: this residue is also strictly conserved in all BVMOs and it interacts with the adenine base and with the phosphate of NADPH [114].

A summary of the conserved residues and motifs can be found in Table 2. In **Paper I**, the analysis of the conserved residues considered all the recombinantly expressed BVMOs for which information was available.

Another interesting analysis of conserved sequence features among BVMOs has been proposed by Rebehmed and co-workers [114]. This analysis considered 116 protein

sequences of which the great majority is not expressed and characterised, against the 60 of **Paper I**, but they belong to only three of the seven BVMO groups proposed in **Paper I**. Therefore, only a limited pool of BVMOs have been taken into consideration for this analysis.

Table 2: Residues and motifs that are conserved by over 90% of the BVMO sequences considered in the alignment.

| Motifs identified | | Position according to amino acid sequence of CHMO from <i>Acinetobacter</i> sp. NCIMB9871 | Description |
|--|-----------------------|---|------------------------------|
| In Paper 1 | Previous studies | | |
| GXGXXG | GXGXXG | 13 -18 | First Rossmann [111] |
| GXWXXXXYPGXXD | A/GGGXWXXX[F/Y]PGMXXD | 43 – 57 | BVMO motif |
| PXXGXGXXFXGXXXHXXXW | FXGXXXHXXXW[P/D] | 150 – 168 | BVMO motif [111] |
| GXGXX[G/A/S] | GXGXX[G/A] | 180 – 185 | Second Rossmann [111] |
| DX[I/L][V/I]XXTG | - | 372 – 379 | ATG motif [116] |
| PN(X) ₄₋₅ G (G is not conserved in CPDMO group) | - | 420 – 427 | PN(X) ₄₋₅ G motif |
| Conserved residues which are not part of motifs | | | |
| W (except BVMO3 of <i>Dietzia</i> sp. D5) | | 122 | |
| G | | 142 | |
| R | | 207 | NADPH interaction [117] |
| P (A in CDMO) | | 320 | |
| R | | 327 | Catalytic residue [113] |
| G | | 394 | |
| G | | 413 | |

Sequence similarities often reflect the evolutionary and functional similarities. Sequences with close similarity often catalyse similar reactions. The BVMO sequences from different organisms have been analysed and are found to cluster into groups according to the similarity of their protein sequences. Several subdivisions of BVMOs

have been proposed in different reports [82, 93, 115] and yet another subdivision is suggested in **Paper I** and seven groups were identified (Figure 15).

The BVMO groups that were identified in the protein sequence analysis made in **Paper I** were named after a known BVMO in the group. The groups are named as: Phenylacetone monooxygenase (PAMO), Cyclohexanone monooxygenase (CHMO), Cyclopentanone monooxygenase (CPMO), Cyclopentadecanone monooxygenase (CPDMO), Ethionamide monooxygenase (EthA), 4'-Hydroxyacetophenone monooxygenase (HAPMO) and Group VII where there is no well characterised member. A brief description of some of these groups is given below.

BVMOs from *Dietzia* sp. D5 belong to Group VII (BVMO1 and BVMO2), EthA group (BVMO3) and CPDMO group (BVMO4).

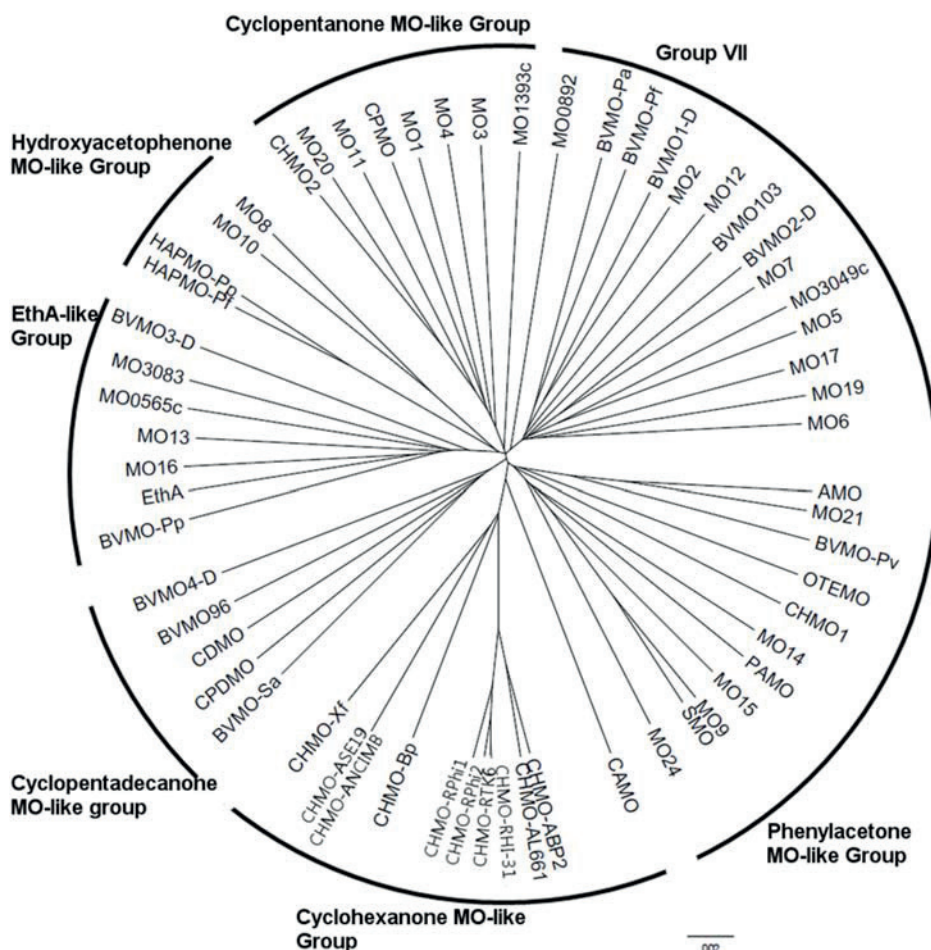


Figure 15: Subdivision of BVMOs in groups according to sequence similarity features.

3.2.3 The different BVMO groups

Generally, enzymes with high sequence similarity are expected to oxidise similar types of substrates. In the case of BVMOs, the substrate promiscuity makes this assumption more complicated. However the clustering may suggest that the members may share some similar catalytic properties. Hence, the description of the representative BVMOs from the groups is given below with the assumption that the properties at least to some extent are shared by the group members.

3.2.3.1 *Cyclohexanone monooxygenase (CHMO)*

CHMO from *Acinetobacter calcoaceticus* NCIMB 9871 is one of the first BVMOs ever discovered [69], cloned and sequenced [118]. The activity of this enzyme has been characterised in depth and it is known to efficiently oxidise cyclic ketones and sulfides [69, 119-120]. Since a number of fundamental studies have been conducted on this enzyme, CHMO is of great importance among all the BVMOs and a number of fundamental studies have been conducted on this enzyme. In addition, large scale production of the enzyme [121] and its pilot scale application for the production of a chiral lactone in a 200 liter scale fermenter has been demonstrated [122]. Initial information on the active site of this enzyme was obtained by analysing the reaction efficiency with a number of substrates with different structures [123-124]. Moreover, mutagenesis studies of CHMO were done using different strategies that resulted in the inversion of the stereopreference for several products [125-126] as well as increased stability to oxidative stress and temperature [127-128]. Although the three-dimensional structure of this CHMO was never published, it was possible to determine a homology model based on available structures, especially using the X-ray structure of the closely related CHMO from *Rhodococcus* sp. HI-31 [117, 129].

3.2.3.2 *Phenylacetone monooxygenase (PAMO)*

PAMO is the first and at the moment the only thermostable BVMO ever reported. This enzyme was isolated from a thermophilic microorganism *Thermobifida fusca*. It is active even at 70 °C and exhibits a half-life of one day at 52 °C [80]. Soon after its discovery the crystal structure of the enzyme was published which was the first BVMO structure experimentally determined [73]. Although this structure is missing the cofactor NADP⁺, an updated version of the structure has been recently published [72]. The enzyme in this updated structure binds both FAD and NADP⁺ and has a 2-(N-morpholino)ethanesulfonic acid (MES) molecule in the active site, that was speculated to adopt a position occupied by the substrate during catalysis. In addition to its stability at high temperature, the enzyme is tolerant to organic solvents and it outperformed the other BVMOs with which it was compared to [130-131]. However, the substrate scope of PAMO is restricted and mutation studies have addressed this problem. A single mutation of the methionine 466 to a glycine resulted

in great changes in the substrate preference as well as stereoselectivity of the enzyme [132]. The best substrates for PAMO and its mutated versions were ketones with an aromatic moiety and molecules containing a sulfides [133-134].

3.2.3.3 *Cyclopentanone monooxygenase (CPMO)*

Similarly to CHMO, CPMO was one of the first BVMOs ever discovered and characterised [70] and it is part of an operon for the catabolism of cyclopentanol in *Comamonas* sp. [75]. As the name suggests, this enzyme is especially active towards cyclopentanone, however it is considered to be one of the BVMOs with the broadest substrate scope [135-136]. In fact, this enzyme has been used as a template to expand the substrate scope of the more chemoselective PAMO [137].

3.2.3.4 *4'-hydroxyacetophenone monooxygenase (HAPMO)*

HAPMO group counts only four members of which two have been purified and characterised in detail [138-139]. Both these enzymes are isolated from *Pseudomonas* spp. and one of them was postulated to be involved in the degradation pathway of 4'-hydroxyacetophenone. These enzymes convert 4'-hydroxyacetophenone and other substituted version of acetophenone with a different degree of activity, although it cannot oxidise other typical BVMO substrates, such as cyclohexanone and cyclopentanone [138-139]. One peculiar characteristic of these enzymes is an extension in the N-terminal sequence of about 100 amino acids that is not present in most BVMOs, with the exception of cyclopentadecanone monooxygenase, although CPDMO one is shorter and non homologous to HAPMO's N-terminal. Truncation of the N-terminal resulted in the expression of inclusion bodies and inactive proteins. The structure of these proteins is not available and it is yet unknown what is the function of this long N-terminal part of the protein [139].

3.2.3.5 *Ethionamide monooxygenase (EthA)*

EthA was identified from the pathogenic organism *Mycobacterium tuberculosis* H37Rv [140-141]. It was demonstrated that EthA was responsible for the oxidation of the pro-drug ethionamide into its bioactive form. Therefore this gene and its regulator (EthR) have been the target of medical studies to understand the resistance of the bacterium to this drug and as a consequence, information on the residues necessary for the enzyme activity has been determined [142-143]. EthA has also been cloned and characterised which revealed that it oxidises aliphatic ketones (C₆ to C₁₂) as well as ethionamide [144]. Nevertheless, the study of EthA did not progress further, possibly because of its low stability in comparison to other BVMOs [130].

In **Paper II**, a BVMO from *Dietzia* sp. D5 with remarkable sequence similarity to EthA, called BVMO3 is presented. Like EthA, it could oxidise long chain aliphatic ketones effectively and the conversion of these and other substrates was performed

using growing as well as resting cells and crude cell extract. However, in contrast with EthA, BVMO3 could not oxidise ethionamide. In order to elucidate the origin of such difference, an analysis of the sequence and secondary structure of BVMO3 with other similar enzymes as well as a comparison of the catalytic activities of sequence related enzymes was provided. Unfortunately, the enzyme lost its activity during purification and showed poor stability when prepared as crude cell extract. It remained active only in some buffers and storage conditions for a few weeks.

3.2.3.6 Cyclopentadecanone monooxygenase (CPDMO) and cyclododecanone monooxygenases (CDMO)

The ability of oxidising bulky cyclic ketones by BVMOs was initially discovered in the organism *Rhodococcus ruber* [145]. Shortly after this finding, a gene cluster from this organism was cloned, among which was the genes encoding Cyclododecanone monooxygenases (CDMO) [146]. The expression of the genes cluster resulted in the oxidation of cyclododecanone to the respective ω -hydroxyacid, 12-hydroxydodecanoic acid. Bulky cyclic ketones (C₁₁ to C₁₅) were favoured over smaller and medium ones (C₆ to C₁₀). The genes in the cluster indicated that CDMO is part of a catabolic pathway for the degradation of bulky cyclic hydrocarbons that are finally transformed into metabolites for the β -oxidation pathway.

Like CDMO, CPDMO efficiently oxidises bulky substrates. As the name implies, one of the substrate for which CPDMO had the highest activity was cyclopentadecanone [147]. These enzymes showed great potential in biocatalytic applications, such as for the oxidation of steroids [148] and a number of other ketones, with different regio- and stereoselectivity as compared to other known BVMOs [115, 135, 149-150].

In **Paper III**, a BVMO that is closely related with CPDMO was cloned, expressed and characterised. However, despite the relatively high sequence similarity, BVMO4 substrate preference and gene cluster organization differ from that of CPDMO and CDMO. In fact, it seems that BVMO4 is not part of a gene cluster similar to the one for CDMO which is involved in the primary metabolism of hydrocarbons [146]. Search for BVMO4 like enzyme in other *Dietzia* spp. genomes revealed that it exists only in *Dietzia* sp. D5. In fact, by comparing the genome of *Dietzia cinnamea* with the one of *Dietzia* sp. D5, it appears that BVMO4 originates from a DNA insertion or deletion at the genome level in one of these organisms. In addition, unlike CDMO and CPDMO, BVMO4 cannot oxidise bulky cyclic ketones. BVMO4 oxidises a number of other substrates such as phenylacetone, bicycloheptanone, 2- and 3-methylcyclohexanone. It is also fairly stable with a half-life of 20 hours at 35 °C, and can be stored for months at -20 °C with practically no loss of activity.

3.3 Heterologous expression of BVMOs

Over the years, the recombinant DNA technology has become a common tool to facilitate the production of enzymes in heterologous hosts and today this is the method of choice to study BVMOs, rather than the tedious process of isolating enzymes from wild type organisms.

Generally at the laboratory scale, the organism of choice for the expression of BVMOs is the common workhorse *E. coli*. However, *Saccharomyces cerevisiae*, *Corynebacterium glutamicum* and *Staphylococcus carnosus* have also been used as hosts for recombinant expression of BVMOs [151-153]. For instance, a recombinant BVMO was nicely expressed in soluble form in *C. glutamicum*, but the final concentration of the biotransformation product in a fed-batch reactor was not outstandingly higher than the one achieved in *E. coli*, hence this approach was not investigated further. In the case of *S. cerevisiae*, the recombinant protein proved to be an efficient biocatalyst when used in whole-cell preparation [154]. Nevertheless, a comparative analysis between the expression of a BVMO in *S. cerevisiae* and *E. coli* showed that *E. coli* is a better option if the interest is the purification and characterization of the protein due to the low expression yield and abundance of proteases in the yeast [155]. In the case of *S. carnosus*, the initial work on the expression of a BVMO was established but expression of the active enzyme has not been achieved yet. Further experiments are expected to troubleshoot the expression of an active BVMO in this microorganism [153].

E. coli is by far the best option for lab scale expression of recombinant proteins because many strains as well as plasmids are available in the market, a lot is known about its physiology and it is easy to cultivate. Although there are BVMOs which are expressed successfully in *E. coli*, there are also several reports that document difficulties in expressing functional BVMOs in *E. coli* systems [87, 138, 156]. The reasons behind this problem are several (e.g. intrinsic instability of the enzyme, proteolytic cleavage, possible interactions of the enzyme with cell membrane and incorrect folding of the protein) and different strategies have been used to achieve the expression of active recombinant BVMO.

The *E. coli* strain in which the enzyme is expressed seems to play an important role. *E. coli* derived from the K12 and the B strains have been used [76], but the most commonly used strains are BL21(DE3) and some other strains derived from it such as Rosetta(DE3), Rosetta2(DE3) and RosettaGami(DE3). In particular, the Rosetta strains can be advantageous because they contain a plasmid for the expression of additional tRNAs that are rare in the *E. coli* genome but that are common in genes of other organisms. The lack of these tRNAs in *E. coli* can strongly impair the expression of the recombinant gene. These types of strains were successfully used in some reports [82, 138].

A BVMO from *Pseudomonas fluorescens* DSM 50106 was co-expressed with chaperones which resulted in active and properly folded enzyme [156]. Although there are more than one case where chaperones are used for successful expression of BVMOs [157], the co-expression of chaperones is not a universal solution to the problem of low soluble expression level of BVMOs [87, 138].

The plasmid in which the recombinant gene is cloned can also play a role on the successful protein expression. The intrinsic differences in the plasmid itself and also the induction system used to start the transcription of the recombinant gene influence the expression of the recombinant protein [93]. Finally the cultivation conditions, such as culture media, temperature, shaking speed, addition of cofactor precursors, etc. can influence the outcome of the protein expression.

In **Paper I**, **Paper II** and **Paper III** the abovementioned variables were taken into consideration to express the recombinant *Dietzia* BVMOs. In particular, the expression of BVMO3 (**Paper II**) was achieved after a long optimization procedure, in which different strains, culture media, inducer and temperature were tested to increase the otherwise low level of active soluble protein. The most important improvement in the expression of BVMO3 was achieved using *E. coli* BL21 CodonPlus(DE3)-RP as the expression host. This strain is also the choice for BVMO4 expression (**Paper III**, **Paper IV**, **Paper V**). Among the four BVMOs of *Dietzia* sp. D5, it was BVMO4 that outclassed the others for its level of soluble expression.

Another strain of *E. coli*, *E. coli* ArcticExpress(DE3)-RP has also been considered for the expression of *Dietzia* BVMOs (**Paper I**, **Paper II**, **Paper III**). This strain is an improved version of *E. coli* BL21 CodonPlus(DE3)-RP that contains in addition a plasmid for the expression of psychrophilic chaperones which are assumed to help the proper folding of the target enzyme. The expression of the chaperones is induced by low temperatures (10-12 °C) and should be maintained throughout the expression phase to allow the chaperones to function properly. In our experience, the low temperatures and the chaperones are of help in the expression of an active BVMO; however, the low temperature drastically reduces cell growth and concomitantly the recombinant protein yield.

3.4 Three dimensional structures of BVMOs

To date, the structures of only four BVMOs have been experimentally determined [73, 117, 158-159]. The intrinsic instability and flexibility of the enzymes which may affect the crystallization can partly explain why there are few structures determined

for BVMOs. Attempts to crystallise *Dietzia* sp. D5 BVMO4 were made and several crystallization conditions have been tried with no success.

BVMO structures contain two domains, FAD and NADPH domains, connected by linkers. The relative position of the two domains changes throughout the catalytic cycles and can swing between two conformations called “open” and “closed”. It was suggested that the “closed” conformation is the one assumed starting from the reduction of the flavin and during the following catalytic steps up to the release of NADP⁺, the enzyme shifts towards the “open” conformation [117].

These structures helped to elucidate the position of conserved residues which are important for catalysis and binding of the cofactors. The available structures also serve as a springboard for *in silico* determination of BVMO structures through homology modeling [114, 160-161].

Homology modeling is a useful tool that can provide a prediction of structural information about a protein for which there is no available 3D-structure. Nevertheless, the outcome of such prediction depends on several factors (such as the algorithms used for the calculation, sequence identity between the query and the template and refinement of the calculations) and it can result in an accurate model or an in a not accurate one.

In **Paper V**, a homology model for BVMO4 is generated using structures retrieved from Protein Data Bank (PDB) as templates. The template proteins sequence identities were in the range of 30% and 27%, and the best coverage of the final homology model of BVMO4 was 86% of the whole sequence length. In fact, the N- and C-termini of BVMO4 sequence were not included in generating the model. Two homology models have been proposed, one for the open and one for the closed conformation. The model includes docked NADPH and FAD in the protein structure (Figure 16). The model was validated and used to select mutation sites to engineer the enzyme (**Paper V**).

3.5 Engineering of BVMOs

Protein engineering is a powerful tool used to improve the properties of enzymes (e.g. enhance their activity towards target substrates, and improve stability against the effect of temperature, solvents, pH, etc.). Mutagenesis can be done using different strategies involving both random and site specific mutagenesis approaches. The choice of the method of engineering mainly depends on two factors: the availability of sequence and structural information and the screening method used and its capacity.

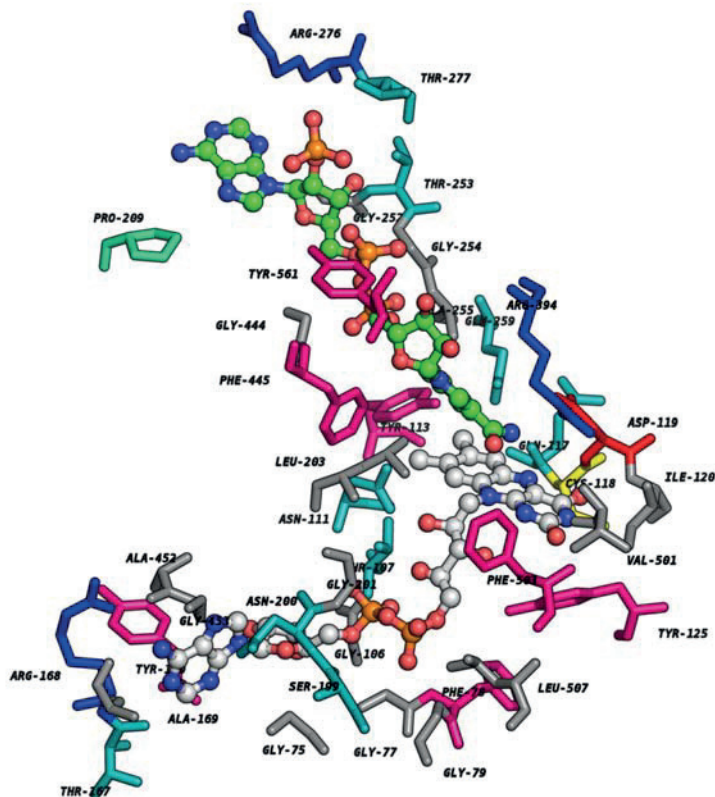


Figure 146: Residues in the proximity of FAD (white stick and balls) and NADPH (green stick and balls) in BVMO4 homology model. Residue coloring depends on the nature of the residues: hydrophobic = grey, aromatic = pink, polar = teal, positive = blue, negative = red, cysteine = yellow and proline = green.

When the structure of the enzyme is available and the reaction mechanism is elucidated, it is relatively easy to fine tune the activity of the enzyme by targeting specific amino acids that play a role in determining the substrate specificity or other properties of interest. On the contrary, when the protein structure is not known or the reaction mechanism is not clearly elucidated a random approach can be pursued, in which mutations are randomly introduced in the gene encoding the enzyme by e.g. error prone PCR. Then the enzyme variants produced are tested to verify if the desired property is acquired. In order to increase the possibility of picking a mutant with the desired property, in the random mutagenesis approach it is necessary to screen a large number of clones. Therefore a screening method that is easy to prepare and read (e.g. colorimetric methods) is preferred to more time consuming techniques.

A compromise between these two approaches, random and site-directed mutagenesis, is site saturation mutagenesis technique. Similar to the site directed mutagenesis approach, it targets specific amino acids, but these sites are mutated into random amino acids. In this case, only approximate structural information of the enzyme are necessary and the library size is limited to tens or hundreds of clones, rather than thousands to screen in the random mutagenesis approach.

Several studies have reported engineering of one of the well studied and stable PAMO with different strategies: site directed mutagenesis [132, 137], site saturation mutagenesis [162-163] and evolution of the saturation mutagenesis techniques, such as multiresidues iterative saturation mutagenesis [164] and OmniChange [165]. Another BVMO whose features have been modified through mutagenesis is CHMO from *A. calcoaceticus*. Also in this case site saturation mutagenesis [166], site directed mutagenesis [167] as well as directed evolution [125, 168] were used.

Other than CHMO and PAMO, only three other BVMOs were mutated to change their substrate specificity. Directed evolution was applied to increase the enantioselectivity of a BVMO from *Pseudomonas fluorescens* towards 4-hydroxy ketones [169]. In another case, the elucidation of the protein structure of steroid monooxygenase (STMO) from *Rhodococcus rhodochrous* by X-ray crystallography allowed the use of site directed mutagenesis approach to study the structural basis of substrate preference [159]. Site saturation mutagenesis has been carried out on cyclopentanone monooxygenase (CPMO) from *Comamonas* sp. based on information obtained from the homology model structure of CPMO because there is no experimentally determined structure of this enzyme or of any closely related enzyme [161].

Similar to the CPMO example, in **Paper V** the engineering of BVMO4 is done using site saturation mutagenesis on sites selected using the homology model structure. In addition to the model structure, the sites for saturation mutagenesis have been selected with consideration of mutagenesis results obtained from previously reported studies on other BVMOs. Mainly residues with close proximity to the flavin ring of the FAD cofactor that were not conserved in the majority of BVMOs were targeted in the engineering of BVMO4. In addition, three residues that are not in the vicinity of the cofactor were also targeted. In total, twelve sites were mutated and for each of them a site saturation mutagenesis library was established. The libraries were screened for conversion of cyclohexanone to caprolactone. The screening identified several mutants that show increased cyclohexanone oxidising activity. In particular, mutation of I120 to valine increased the substrate conversion by about 5-fold. Mutation of each of the two neighboring tyrosines at position 499 and 500 resulted in a far greater improvement in conversion of cyclohexanone. Especially, the mutations of Y499 to the residues isoleucine, leucine or phenylalanine yielded a conversion increase between 11 and 12-fold compared to the wild type.

3.6 Applications of BVMOs in biotransformations

Although there are a wide variety of oxidative enzymes that have been studied, the number of enzymes that made its way to catalyse industrial redox reactions is still limited. This lagging is even more pronounced in the case of BVMOs despite their interesting biocatalytic features.

In the last four decades, a large number of BVMOs have been characterised and their application potentials have been demonstrated. For instance, in asymmetric Baeyer–Villiger and heteroatom oxidation the use of BVMOs is well ahead compared to their chemical counterparts. However, despite their enormous potential in biotransformation applications, BVMOs have been sparingly utilised at industrial scale, so far.

BVMOs are promiscuous enzymes that catalyse the oxidation of a wide variety of substrates, and this is interesting from an application point of view as it allows to oxidise a wide variety of substrates. Initially, BVMOs were known for the oxidation of ketones to the corresponding esters [69, 71]. A number of molecules containing keto-groups are reported to undergo oxidation by BVMOs. Cyclic ketones, from cyclobutanone up to the bulky cyclopentadecanone, are known to be substrates for different BVMOs [82, 93, 147]. Resolution of racemic mixtures of alicyclic ketones substituted at different positions have also been reported, and different regio- and enantioselectivity is found for different BVMOs [136, 170-171]. In particular, this kind of activity is interesting for the production of chiral and achiral lactones and aroma compounds [149, 172-173].

Keto-groups in bicyclic molecule such as bicyclo[3.2.0]hept-2-en-6-one are also substrates for BVMOs. This type of substrates is of particular interest because the different regio- and enantiomers can be used as a precursor in the synthesis of non-natural nucleosides and prostaglandins [174]. In fact, the first industrial application of a BVMO was realised by Sigma Aldrich where bicyclo[3.2.0]hept-2-en-6-one is oxidised to yield (-)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one and (-)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one.

Moreover, heterobicyclic ketones are also accepted as substrates by BVMOs and their oxidation could yield molecules to be used in the synthesis of natural compounds with antibiotic and cytostatic activity [175]. BVMOs have also been tried to synthesise compounds of interest to perfume industry. In fact it was evaluated the production of enantiopure jasmine lactones which resulted in high yields and excellent *ee* values by CPDMO and another BVMO [150].

In addition to cyclic molecules, linear aliphatic ketones can also be oxidised by BVMOs. The keto-group can be part of an aliphatic molecule or an aromatic moiety that is a substituent: an example of the latter case is phenylacetone. For instance,

PAMO and HAPMO readily oxidise phenylacetone and hydroxyacetophenone, respectively [176-177]. According to the expectations, the oxygen added during the BV reaction is inserted in the more substituted side of the molecules, which in the case of phenylacetone would be between the ketone and the phenyl group. Enantioselective reactions with this type of substrates is also possible if a substituent is added in the aliphatic part of the molecule [133, 178] and regiospecific oxidations were performed on 3-phenylpenta-2,4-dione [134].

Table 3: Conversion of aliphatic ketone substrates by BVMO3. Whole-cell biotransformations with growing and resting cells and crude cell extract were performed with 5 mM substrate, while biotransformations with crude cell extract contained 25 mM substrate and glucose/glucose dehydrogenase for cofactor regeneration. The ratio of a:b is reported in brackets. NC stands for not converted; ND stands for not determined.

| Substrate | Product | Conversion | | |
|--------------|---------|----------------|----------------|--------------------|
| | | Growing cells | Resting cells | Crude cell extract |
| 2-Heptanone | | 41.1% | Traces | 73.6% |
| 2-Octanone | | 60.8% | 6.6% | 78.3% |
| 2-Nonanone | | 89.8% | 3.2% | 82.4% |
| 2-Decanone | | 80.8% | 10.5% | 83.7% |
| 2-Undecanone | | 63.9% | NC | 57.9% |
| 3-Octanone | a | 44.3% (1:1) | 5.9% (2:3) | 43.6% (1:1) |
| | b | | | |
| 3-Nonanone | a | 64.2% (1:1) | 1.6% (2:3) | 45.9% (1:2) |
| | b | | | |
| 3-Decanone | a | 85.9% (2:1) | Traces (ND) | 59.0% (1:1) |
| | b | | | |

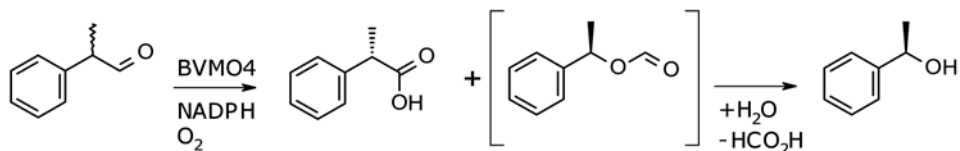
Linear aliphatic ketones can be transformed into esters by some BVMOs. In this case, regioselectivity of the oxidation can vary depending on the property of the enzyme, the substrate carbon chain length and position of the keto-group. In general, the oxygen is inserted in the most substituted position. Nevertheless, there are examples in which the unfavoured regioisomer is also produced [144, 157, 179]. A summary of the BVMOs capable of oxidising linear aliphatic ketones and their substrate scope is

presented in **Paper II**. In fact, BVMO3 proved to be an interesting enzyme for the oxidation of linear aliphatic ketones, from C₇ to C₁₁, especially 2-nonanone and 3-decanone (Table 3).

In addition to ketones, BVMOs such as HAPMO, PAMO and CHMO can also oxidise aldehydes although the studies have been done with a very limited number of substrates [119, 133]. In **Paper IV** the conversion of a number of substrates containing an aldehyde group by BVMO4 is reported. This enzyme could convert some of the aldehydes tested, in particular those with an aromatic moiety and linear aldehydes. Most importantly, the regioselectivity of BVMO4 is opposite to the one reported for PAMO and HAPMO, as the most abundant oxidation product is the carboxylic acid rather than the formate ester (Figure 17).

In addition to carbonyls, BVMOs have been reported to be capable of oxidising heteroatoms to the corresponding oxide [119]. Although the majority of the reports focused on sulfides [134, 180-184], there have been reports on BVMO catalysed oxidation of selenides, boronic acids, phosphite esters and iodide ions [119, 185-186]. Sulfoxides are chiral compounds that can be used as chiral auxiliaries [187] and are present in the proton pump inhibitor drugs called prazoles [188-189]. Codexis Inc. has also patented a process including an engineered BVMO for the enantioselective sulfoxidation of a prochiral sulfide to enantiopure prazoles [190].

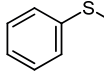
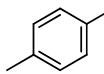
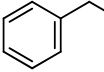
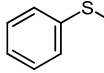
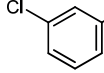
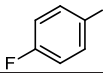
Figure 157: Reaction scheme of 2-phenylpropionaldehyde oxidation by BVMO4.



Among the BVMOs that were shown to react with sulfides, are CHMO from *A. calcoaceticus*, CPMO, PAMO, (HAPMO) and two BVMOs from *Pseudomonas putida* [134, 180, 182-184] but all of these enzymes have low sequence similarity with BVMO4. Therefore, in **Paper IV** the enantioselectivity of BVMO4 was tested using a library of sulfides and compared with available literature data (Table 4). This comparison shows that BVMO4 could oxidise several sulfide-containing substrates with a similar enantioselectivity reported for CHMO, although these two enzymes have low sequence identity (below 25%).

Table 4: Comparison of the enantioselectivity of BVMO4 with previously reported BVMOs and smFMO. smFMO is flavin monooxygenase from *Stenotrophomonas maltophilia* [55], CHMOAc is cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB9871[183], CPMO is cyclopentanone monooxygenase from *Comamonas* sp. NCIMB9872 [180], PAMO is phenylacetone monooxygenase from *Thermobifida fusca* YX [134], HAPMO is 4'-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB [182], 2,5-DKCMO is 2,5-diketocamphane monooxygenase from *Pseudomonas putida* NCIMB10007 [184] and 3,6-DKCMO is 3,6-diketocamphane monooxygenase from *Pseudomonas putida* NCIMB10007 [184]. NR = Not Reported;

* This data was reported by Colonna and co-workers [191].

| Substrate | BVMO4 | smFMO | CHMO Ac | CPMO | PAMO | HAPMO | 2,5- DKCMO | 3,6- DKCMO |
|---|----------------------|---------------------|-------------------------------------|----------------------|---------------------|---------------------|---------------------|---------------------|
|  | 50 % (<i>R</i>) | 21% (<i>R</i>) | 99% (<i>R</i>) | 100% (<i>S</i>) | 44% (<i>R</i>) | 99% (<i>S</i>) | 56% (<i>S</i>) | 11% (<i>S</i>) |
|  | 83 % (<i>R</i>) | 25% (<i>R</i>) | 37%(<i>S</i>) 87%(<i>R</i>)* | 84% (<i>S</i>) | 10% (<i>R</i>) | 99% (<i>S</i>) | 75% (<i>S</i>) | 40% (<i>S</i>) |
|  | 58 % (<i>R</i>) | 24% (<i>R</i>) | 54% (<i>R</i>) | NR | 94% (<i>S</i>) | 85% (<i>S</i>) | NR | NR |
|  | 99 % (<i>R</i>) | 71% (<i>R</i>) | 47 % (<i>R</i>) | NR | 55% (<i>S</i>) | 99% (<i>S</i>) | 8% (<i>R</i>) | Racemic |
|  | 17 % (<i>R</i>) | 15% (<i>S</i>) | NR | NR | NR | 96% (<i>S</i>) | 57% (<i>S</i>) | 29% (<i>S</i>) |
|  | 88 % | NR | 92% (<i>R</i>) | 92% (<i>S</i>) | NR | NR | NR | NR |

4. Challenges and opportunities in the application of BVMOs

In the previous chapter, it has been indicated that despite BVMOs great potential for industrial applications, so far, very little has become an established practice. As there are many interesting catalytic properties demonstrated, there are also a number of drawbacks that contributed to limit BVMOs application in industry. Indeed, great progresses in the field of BVMOs have been made in the last forty years, since the first enzymes were discovered and characterised [69-71]. Nevertheless, there are still challenges that need to be properly addressed so that the remarkable potential of these enzymes will be utilised in broader scale. The most important challenges that hindered application of BVMOs are discussed below.

4.1 Lack of ideal BVMOs

Enzymes intended for industrial application should be robust and remain active at the required process conditions and during storage. Ideal BVMOs are enzymes that are stable, resistant to high substrate/product concentration, easy to produce and not hampered by difficult and expensive co-factor recycling systems..

4.1.1 Stability

As stated above, BVMOs intended for industrial application should be stable during storage and catalysis. Unfortunately, the great majority of BVMOs reported so far are not stable and rapidly lose activity even after few days of storage at 4 °C [62, 192]. In fact, the poor stability of BVMOs is one of the greatest hurdles to overcome for their industrial application [76, 193]. Thus, it is of great interest to develop stable BVMOs.

Different strategies can be considered to develop stable enzymes that are more suitable for the intended application. Screening of organisms from unexplored habitats, especially extreme environments, can potentially provide access to novel stable enzymes. Enzymes from extreme environments are often found to be resistant to harsh conditions, such as those that can be found in industrial processes. For instance, the only thermostable BVMO, PAMO, originates from a thermophilic organism

Thermobifida fusca and shows a remarkable operational stability at fairly high temperatures [80]. This suggests that screening of extremophiles can potentially lead to stable BVMOs. In this thesis work four interesting BVMOs were identified from a newly isolated organism, *Dietzia* sp. D5, that was isolated from a soda lake sample (**Paper I**, **Paper II**, **Paper III**). With respect of stability, one of the four *Dietzia* sp. D5 BVMOs, BVMO₄, is very interesting as its characterization revealed that it is probably the second most stable native BVMO ever reported (**Paper III**).

In addition to the screening of microorganisms for stable enzymes, existing BVMOs can be engineered to suit industrial requirements. However, very little is known about what causes the rapid inactivation of most BVMOs. High flexibility of the enzymes that is necessary to bind and release NADPH as well as the substrate might be one of the factors contributing to the instability. Thus, improving the stability of BVMOs by protein engineering is challenging, however, there are reports where the stability of BVMOs was increased by gene manipulation. For instance, researchers at Codexis increased the stability of a BVMOs by 10,000-fold through mutagenesis of the BVMO gene encoding gene [190]. Insertion of disulphide bridges has also been demonstrated to increase the stability of CHMO, possibly by increasing the rigidity of the protein [128]. Another successful strategy investigated is the combination of scaffolds from the thermostable PAMO with other BVMOs [194]. The chimeric enzymes exhibited higher thermostability than the wild type enzyme.

In principle, great improvement on stability can be achieved through several cycles of directed evolution, a very powerful method that creates an amazing diversity but it also needs efficient screening system. The limitation of this method is not only the time but also the high costs associated with screening of the large library, which could be challenging for smaller companies with limited resources.

However, obtaining a stable enzyme is not the arrival point *per se*: no stable enzymes are useful if their substrate scope is limited. In fact, this is the case for the thermostable PAMO, that is considered to have a narrow substrate scope. Several studies were conducted to increase the substrate acceptance of PAMO by means of enzyme engineering and encouraging results were obtained [132, 137, 162-165].

BVMO₄ has a quite wide substrate scope, however it does not react or reacts poorly with cyclic ketones. Therefore, BVMO₄ was engineered to enhance its activity on oxidation of cyclohexanone to caprolactone (**Paper V**). This study resulted in mutants with highly enhanced conversion of cyclohexanone. It has been possible to achieve up to 12-fold improvement in the conversion of cyclohexanone. Since it is the first engineering work performed on BVMO₄ or any other closely related BVMOs, the data generated is useful for future improvement studies. Moreover, combining the mutations that gave a positive outcome could help to further increase the conversion of cyclohexanone by BVMO₄.

In general, although the knowledge on BVMOs catalytic mechanism has significantly increased in the last decade, many aspects such as the conformation of the active site during catalysis or the exact position of the substrate in the active site are not yet fully understood and detailed elucidation of the structure-function relationship of the active site area would help greatly to engineer BVMOs successfully [38].

4.1.2 Substrate and product inhibition

Industrial processes are often done at high concentration of substrates and hence enzymes intended for large-scale applications should remain active preferably at high substrate and product concentration. Unfortunately, the great majority of BVMOs available to date are active only at extremely low substrate concentrations (1 to 10 mM), making it unattractive for most industrial processes. Recently, it was showed that the low substrate concentration are needed since BVMOs suffer greatly from substrate and product inhibition and their stability is also affected at high substrate and product loads [172, 195-196]. Thus, BVMOs that do not suffer of such limitation are of great interest.

Another reason to implement BVMOs that can tolerate high concentration of reactants is that low product concentration poses downstream processing (DSP) problem since the product recovery from a large reaction volume is costly [51, 197]. Moreover, such DSP would generate a high amount of waste which has to be handled properly. Process analysis such as Life Cycle Assessment, or even the simpler E factor calculations [198], could indicate the process parameters that should be adopted to be adherent to the Green Chemistry Principles, since the use of enzymes to catalyse a reaction does not make by itself a process “green” [51].

Several strategies, other than engineering of the enzyme, can be employed to alleviate problems related to substrate concentration and product recovery. For example, the use of resins that absorb the substrate, the product or both, can allow increasing the substrate load. The use of the resin makes the overall free substrate/product concentration in the reaction media below the inhibitory level and the product will be easily recovered by extraction from the resin [195, 199]. Similarly, it can be used a two phase reaction system where the substrate and/or the product is concentrated, for example, in the organic phase and the enzymatic reaction takes place in the aqueous phase. This reduces the concentration of substrate/product in the aqueous phase, reducing enzyme inhibition, and facilitates product recovery from the non aqueous phase where the concentration is higher [147].

In addition, novel enzymes tolerant to high reactants concentrations can also be obtained through screening of new enzymes or engineering the existing BVMOs. For instance in this thesis, the characterization BVMO3 revealed that this enzyme can oxidise substrates at an initial concentration of 25 mM (**Paper II**). The activity of the

enzyme was quite outstanding at this concentration that is usually inhibitory for most other BVMOs. However, the enzyme was unstable and the investigation of this enzyme did not continue.

4.1.3 Cofactor regeneration

BVMOs require NADPH as a cofactor to perform the oxidation catalysis and this cofactor has to be regenerated. A number of regeneration systems that reduce NADP⁺ to NADPH have been proposed [200].

The most common regeneration strategies are enzymatic systems. In whole-cell biotransformation process the inbuilt cofactor regeneration machinery is utilised. However, such *in vivo* recycling of the cofactor can be a metabolic burden for the cell leading to insufficient NADPH regeneration. For this reason, it has been investigated the co-expression of cofactor recycling enzymes to alleviate the burden and achieve higher yield [201-202]. In cell-free setups, cofactor recycling enzymes such as formate- or glucose dehydrogenase are often added to the reaction mixture or expressed in a “self-sufficient” form together with the BVMO [196]. Other systems that use light-dependent processes or chemical mediators have also been investigated [203-204]. The choice of the system for cofactor regeneration has to consider if there are any side reactions, if the regeneration system is compatible and efficient, if the cost of the cofactor regeneration is acceptable, as well as that the co-products generated by the regeneration system have to be dealt with in the DSP, and if it is possible to use such a system at the scale required for industrial applications.

Another issue related to the cofactor is the strictly NADPH dependence of BVMOs. NADPH is ten time more expensive than the closely related NADH therefore using the latter would be more economical. However, most BVMOs do not accept NADH as cofactor, while few BVMOs can use NADH for reduction of flavin, although with extremely low efficiency. Some FMOs that can use both NADPH and NADH have been reported [54-55]. Despite the difference between the NADPH binding site of BVMOs and NADH-dependent FMOs is minimal, changing the cofactor specificity is not a simple task [205]. The search for new NADH dependent-BVMOs or engineering of existing BVMOs into NADH dependent enzyme would definitely be interesting if large scale application is considered.

4.1.4 Enzyme expression and availability

Usually enzymes used at industrial scale are produced in very efficient heterologous system to minimise production costs and maximise yields. Many expression systems have been developed for different enzymes. The expression of BVMOs reported so far

are performed mostly in *E. coli* systems and the expression is sometimes troublesome. This shows the need for development of efficient BVMO expression systems. For instance, in this thesis BVMO2 was expressed as inclusion bodies while BVMO1 and BVMO3 were also poorly expressed (**Paper I**, **Paper II**). Changing the strain and the plasmid in which the BVMOs were expressed improved only slightly the expression. BVMO4 constituted an exception to the other *Dietzia* BVMOs (**Paper III**) and its expression was readily available, although in a limited number of *E. coli* expression strains. Availability of alternative expression systems that allow high yield of active protein expression will improve the possibility of using BVMOs at industrial processes. A step forward in this direction has been done recently by the company Enzymicals AG that has established the first commercially available BVMOs, launching in the market a toolbox containing 7 BVMOs with different substrate scopes [206].

4.2 Patents and application directions

A recent (August 2014) patent search on BVMOs resulted in nine patents, excluding those that patented solely the gene sequences. Two of the patents are on non-enzymatic cofactor regeneration methods, two are on production of carboxylic acids (i.e. adipic acid) by microorganisms and the remaining five patents are related to synthesis of commercially relevant chemicals, namely prazoles [190, 207-208], fragrances [209] and lipoic acid [210]. The fact that only five patents on BVMO catalysed synthesis have been deposited shows clearly that there is a gap between the industry needs and the development of BVMOs.

BVMOs can catalyse the oxidation of many substrates with high regio- and stereoselectivity. Some of the oxidation products are value-added molecules (e.g. prazoles) while others are relatively cheap compounds (e.g. caprolactone). At the current state of development, the production of high value chemicals seems to be the target applications for BVMOs.

Based on the number and type of patents filed, it seems that sulfoxidation reaction is an interesting application due to better enantiospecificity of BVMOs than chemical catalysts and the products are of interest for pharmaceutical industries [190]. In this thesis, the activity of BVMO4 on different sulfide and aldehydes was screened, and the results are encouraging (**Paper IV**). The screening of BVMO4 activity on less complex sulphide containing compounds, revealed that the enzyme could oxidise some of the substrates tested. In some cases, satisfactory enantiomeric excess was obtained in favour of the (*R*) enantiomer (**Paper IV**). Moreover, screening of aldehyde containing substrates revealed that BVMO4 preferably oxidises substrates with a linear aliphatic moiety and some of those with an aromatic moiety (**Paper IV**). This

reaction was particularly interesting because the major oxidation product of the reaction was the carboxylic acid, which is expected to be the unfavoured product from a steric and thermodynamic point of view. A similar outcome for such reaction has been reported 25 years ago [4]. Since then aldehydes were rarely used as substrates for BVMOs, and in the few reports available aldehydes were oxidised to esters, meaning that the oxygen was inserted on the side of the more substituted position [133, 176]. It has to be mentioned that in some cases the regioselectivity of BVMO₄ oxidation of aldehydes is only partial and a mixture of acid and ester is found. However it was shown that the regiospecificity could be influenced by varying the reaction parameters, and possibly the outcome of the reaction can be influenced even more by engineering the enzyme. Improving the enzyme to accomplish this reaction might in fact be interesting as one of the products obtained in **Paper IV**, 2-phenylpropionic acid, is a compound closely related to the family of profens, missing only an isobutyl substituent in *para* position of the aromatic ring. Profens are non-steroidal anti-inflammatory drugs sold over the counter. The profen molecule, analogously to 2-phenylpropionic acid contains a chiral center, and although it has been assumed that the drug undergoes racemisation in the body, profens are starting to be marketed as the single bioactive enantiomer. Therefore, development of efficient process for production of the pure active enantiomer could be attractive. Other enzymatic resolutions in order to obtain the desired carboxylic acid have also been proposed as possible processes [211]. However, the enzymatic resolution using the aldehyde as substrate is especially interesting since theoretically it can achieve complete substrate conversion and complete stereoselectivity. In fact, 2-phenylpropionaldehyde racemises spontaneously in slightly alkaline environment, but the carboxylic acid does not, therefore, if the enzyme is strictly selective for the (*S*) enantiomer, it can be obtained as the only product in one reaction step without leaving behind half of the substrate, as it is the case with other approaches. A similar reaction has been shown possible using Horse Liver dehydrogenase [212], and BVMOs might constitute an alternative approach.

Another interesting area of BVMO application could be in the production of large lactones. Lactones which are bigger than the seven membered ring lactone (caprolactone), are very important molecules in fragrance and flavour industry. Due to the inefficiency of the chemical synthesis, large lactones are supplied to the market in limited amount. However, CPDMO and CDMO are known to produce large lactones such as lauryl lactone and pentadecalactone [146, 213]. The presence of larger lactones in biological systems is well documented and exhibit a large spectrum of biological activities and applications as scents, insecticides, antibiotic and cytotoxic compounds [214-216]. The synthesis of these lactones, that often have several stereocenters, with conventional methods is very difficult and hence the enzymatic synthesis, even using BVMOs, could ease the production of some of these interesting compounds.

5. Outlook and future perspectives

BVMOs are a promising group of enzymes that can potentially catalyse different industrial reactions. This has kept the discovery and characterization of these enzymes an ongoing process for about 40 years. Due to some limitations in the enzymes studied so far, an active search for BVMOs that are more suitable for industrial applications is continuing.

The results of this thesis work suggest that *Dietzia* sp. D5 is an interesting source for BVMOs and more generally oxygenases that can be explored for identifying novel enzymes for different applications. Especially, BVMO4 is an interesting enzyme with regard to its easy expression, substrate scope, rare regioselectivity and stability (**Paper III**, **Paper IV**). This encourages further studies on this enzyme. Moreover, more structural and mutational studies will help to further improve the properties of BVMO4. For instance, similar to the study reported in **Paper V**, efforts to improve conversion of other substrates such as large cyclic ketone (e.g. cyclopentadecanone) and increase its regio- and enantiospecificity towards aldehyde substrates, or further increase its stability would be attractive.

In many cases it has been reported that the expression of BVMOs in *E. coli* strains results in either poor expression or end up in inclusion bodies [88, 93, 156-157]. The difficulty of expressing BVMO3 (**Paper II**) as well as BVMO1 and BVMO2 (**Paper I**) has been experienced in this thesis work. Expression in more controlled environments using fermenter is expected to result in better expression yield than the expression in shake flasks. Moreover, developing new expression systems or screening the available expression hosts and plasmids for better expression is expected to remove the existing production bottleneck. In this regard, it would be interesting to improve further protein expression of BVMO3 and express and characterise BVMO1 and BVMO2.

The challenges highlighted in this thesis remain to be solved and further research on these topics will pave the way for application of BVMOs in the fine chemical production in the near future. If the current research pace is maintained, in the long run these remarkable enzymes may even find application in production of bulk low value added molecules such as caprolactone.

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Paper I

Insights into diversity of oxygenases in the draft genome sequence of *Dietzia* sp. D5 and analysis of the Baeyer-Villiger monooxygenases

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Abstract

A *Dietzia* strain capable of growing on different hydrocarbons has been isolated from a soda lake water sample. Analysis of the draft genome of this organism revealed the presence of 28 monooxygenases and 15 dioxygenases. Among these oxygenase encoding genes, four of them are identified as Baeyer-Villiger monooxygenases (BVMOs) and named as BVMO1, BVMO2, BVMO3 and BVMO4. The amino acid sequences of these BVMOs were analysed and compared to other sequences of functionally proven BVMOs. The sequence analysis was made on 60 BVMOs originated from various organisms and led to the formation of seven groups; the four BVMOs from the *Dietzia* strain clustered to three of these groups. Six motifs characteristic of BVMOs were identified from the analysis of which two are identified for the first time. Moreover, group specific fingerprint sequences have been identified almost for each BVMO cluster. An effort has also been made to correlate BVMO sequences to their catalytic behavior; specifically to that of substrate preference. The four BVMOs were heterologously expressed, and BVMO1, BVMO3 and BVMO4 have shown activities. On the other hand, BVMO2 was expressed as inclusion bodies and did not show any measurable biotransformation on substrates tested.

Keywords: monooxygenase; oxidoreductase; *Dietzia*, alkaliphile; Baeyer-Villiger

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Introduction

Baeyer-Villiger monooxygenases (BVMOs) are flavin dependent enzymes that catalyse the oxidation of ketones and different heteroatoms using molecular oxygen as oxidant and NAD(P)H as cofactor. This group of enzymes has been used to oxidise a vast array of compounds, and have shown high chemo- enantio- and regioselectivity for synthesis of molecules, otherwise not possible to achieve by conventional chemistry [1-2]. There is thus a growing interest in developing these enzymes for different applications [3]. Since the degree of substrate preference, catalytic efficiency, selectivity and stability varies significantly among these enzymes, it is useful to have a library of BVMOs from which enzymes suitable for specific applications can be selected. In line with this, a number of BVMOs have been reported from several organisms and over 55 BVMO genes have so far been expressed in heterologous hosts [3]. A great majority of these enzymes are of microbial origin, many of which belong to the bacterial Orders Pseudomonadales and Actinomycetales.

Microorganisms that belong to genus *Dietzia*, a member of Corynebacteriaceae, are related to *Rhodococcus* and *Mycobacterium* which are rich in oxygenases [4]. *Dietzia* spp. are often isolated from hydrocarbon polluted environmental samples [5-9] suggesting that these microorganisms have the ability to metabolise hydrocarbons. In fact, bioremediation of stranded oil spills were found dominated by *Dietzia* strains [6, 9-10] and several of *Dietzia* spp. including *D. cinnamea*, *D. dagingensis*, *D. maris*, *D. psychralcaliphila*, *Dietzia* sp. E1, etc. are known to degrade hydrocarbons [8, 11-12]. The microorganisms that are able to grow on hydrocarbons often have a consortium of oxidoreductases [13]. Among these there are BVMOs which catalyse the oxidation, in aerobic environment, of alicyclic and aliphatic ketones to the corresponding lactones and esters respectively, which is a key step for subsequent metabolic processes [14-15]. Such microorganisms are thus rich sources of potential biocatalysts for oxidation reactions. Recently, Bihari and co-workers [12] have demonstrated the presence of oxygenase in *Dietzia* spp. for the metabolism of long chain n-alkanes.

In the last few years, there has been a growing interest on *Dietzia* spp. in areas such as veterinary [16], bioremediation [9, 17-18], medical [19], food [20] and canthaxanthin and biosurfactant production [21-23], and the genomes of two *Dietzia* spp. have also been sequenced recently [20, 24]. Although related to *Rhodococcus* and *Mycobacterium*, the diversity of oxygenases in *Dietzia* has never been assessed.

Recently, we have sequenced the genome of a *Dietzia* strain isolated from soda lake water sample, which is capable of growing on hydrocarbons such as cyclohexanol, toluene, decanol, catechol and naphthalene. This study explores the diversity of oxygenases identified in the draft genome sequence of this organism and compared it to the diversity found in other organisms' genomes known for their rich oxygenase diversity including two *Dietzia* spp. This work focuses primarily on BVMOs and

deals with analysis and comparison of the *Dietzia* sp. D5 BVMO sequences with other sequences of functionally characterised BVMOs originating from a wide range of organisms. An attempt has also been made to cluster BVMOs based on their sequence similarity and correlate the BVMO clusters with their substrate preferences. Moreover, the BVMOs identified in the genome of *Dietzia* sp. D5 was cloned and expressed in *E. coli* and used for biotransformation of ketones.

Materials and Methods

Isolation of BVMO producing microorganisms

A sample of water from Lake Abjata in the Ethiopian East African Rift Valley was added to sterile nutrient broth (Difco) supplemented with separately sterilised 1% Na₂CO₃. After 48 h of shaking (150 rpm) at 30 °C, a loop full of the culture was streaked on alkaline nutrient agar plates and incubated until colonies were big enough to be picked. The colonies were transferred to fresh plates, and pure isolates obtained after repeated transfers were stored as stock cultures.

The isolates were screened for their ability to grow on minimal media containing different hydrocarbons under aerobic conditions at 30 °C for 5-7 days by shaking at 200 rpm in a rotary shaker incubator. The isolates that were able to grow in cyclohexanone containing medium were used to inoculate 500 ml mineral salt medium (M9 medium) supplemented with 0.2% yeast extract and cyclohexanol (1.5% final concentration, added every 24 h for 5 days). Cultivation was performed at 30 °C with rotary shaking at 200 rpm. The cells were harvested by centrifugation at 6000 × g, and the cell pellet was washed and re-suspended in 20 mM Tris-HCl buffer containing 0.5% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) prior to sonication (8 rounds for 1 min each with intermittent 1 min cooling on ice) using UP 400S sonicator (GmbH, Stahnsdorf, Germany). Cell debris was separated by centrifugation at 10 000 × g (4 °C) and the clear supernatant was used as the enzyme source. The BVMO activity of the supernatants on different substrates (such as cyclobutanone, cyclohexanone, ethionamide, etc.) was tested by monitoring the oxidation of NADPH (160 μM) at 340 nm in a total volume of 1 ml in 50 mM Tris-HCl buffer, pH 8.5 at 25 °C. A background rate of NADPH consumption was measured over 2 minutes prior to the addition of 2 mM substrate to the reaction mixture in the cuvette. Three isolates were found positive for monooxygenase activity and isolate D5 showing the highest activity with cyclohexanone, was selected for further studies.

Genome sequencing and identification of oxygenases

The genomic DNA of isolate D5 was purified using ZR Fungal/Bacterial DNA MiniPrep kit of Zymo Research (Irvine, USA). Genome sequencing and de novo

assembly were performed at BaseClear (Leiden, The Netherlands). Sequencing was done by Illumina using the Iix Genome Analyser (GAIix), and the retrieved sequence reads were analysed using the “de novo assembly” option of the CLC Genomics Workbench version 4.5. On all Illumina GAIix sequence reads, a quality filtering was applied based on Phred quality scores. In addition, low quality and ambiguous nucleotides were trimmed off from the remaining reads.

CLC Main Workbench (CLC bio, Denmark) was used to create local database and identify the target genes. The 16S rRNA gene sequence identified in the genome sequence was compared with other 16S rRNA gene sequences available in public databases using the BLAST algorithm. Multiple alignments of sequences and neighbor-joining phylogenetic tree construction were performed using CLUSTAL W program integrated to MEGA [25]. Screening of the genome for oxygenases was performed by building a local database of all the oxygenases listed under the EC numbers 1.13.x.x and 1.14.x.x in BRENDA (<http://www.brenda-enzymes.org/>). Sequences of oxygenases identified in isolate D5 genome were further confirmed by BLAST search against the NCBI database (<http://www.ncbi.nlm.nih.gov/protein/>) sequences with default settings.

Identification and sequence analysis of BVMOs

The BLAST results for some of the isolate D5 oxygenases revealed high similarities to sequences of BVMOs in the NCBI database, which were analysed further. Database sequences used in the analysis (56 BVMOs) were only those from functionally characterised and proven BVMOs, specifically BVMOs that are cloned and expressed in heterologous systems. The retrieved sequences were aligned together with those of isolate D5 sequences using the multiple sequence alignment program T-coffee [26], an algorithm chosen for its known high accuracy for multiple sequence alignment [27]. Based on the alignment profile, a phylogenetic tree was constructed by the same software and the T-coffee guide tree file generated was visualised with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

The BVMOs were categorised according to the clustering generated by the software. For each cluster, a literature search was made and relevant information about the properties of the enzymes that belong to the cluster was summarised. The theoretical isoelectric points and molecular weights of the BVMOs were calculated using Compute pI/Mw tool available in ExpASY Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/).

Heterologous expression of BVMOs and biotransformations

The BVMO encoding genes identified in the genome of *Dietzia* sp. D5 were amplified by PCR from the genomic DNA using High Fidelity PCR enzyme mix (Fermentas, St. Leon-Rot, Germany). The PCR products were digested with

restriction enzymes and ligated to the expression vector pET22b(+) which was digested with the same restriction enzymes. The ligation mix was transformed to competent *E. coli* NovaBlue cells which were then plated on LB-agar plates containing ampicillin. After overnight incubation at 37 °C, colonies were picked and screened by PCR for the insert (BVMO encoding gene). Recombinant plasmids containing the BVMO gene were sequenced by GATC Biotech (Konstanz, Germany) and plasmids with correct sequences were transformed to competent expression host cells, *E. coli* ArcticExpress(DE3)-RP (Agilent Technologies, Santa Clara, USA). The BVMOs were expressed in *E. coli* ArcticExpress(DE3)-RP cells following the manufacturer instructions. For protein expression analysis, the cells were harvested by centrifugation, re-suspended in lysis buffer and lysed with sonication. The lysate was centrifuged, and samples from the pellet and supernatant were taken and analysed by SDS-PAGE which was performed according to Laemmli [28].

The activity of the recombinant enzymes was checked by adding 5 mM substrate to the culture at the moment of induction of the protein expression by IPTG. After 24 hours of induction and biotransformation at 12 °C, the oxidation of the substrates were monitored by gas chromatography (Varian 430-GC gas chromatograph (Agilent Technologies) equipped with FactorFour VF-1ms 15 m × 0.25 mm (0.25 µm) column (Varian, Strathaven, UK).

Bacterial strain and gene sequences deposition numbers

Dietzia sp. D5 is isolated in our laboratory. The gene sequences for the oxygenases *Dietzia* sp. D5 are available at GenBank NCBI under the accession numbers from KF748495 to KF748536. The 16S rRNA gene sequence is deposited under the accession number KF720331.

Results

Identification of isolate D5

Analysis of the 16S rRNA gene encoding sequence revealed that isolate D5 belongs to genus *Dietzia*, which currently contains 13 described species, and is named hereafter as *Dietzia* sp. D5. The 16S rRNA gene sequence showed a very high similarity to the sequence of *D. cinnamea*. The phylogenetic position of *Dietzia* sp. D5 among the described species of the genus is shown in Figure 1. Similar to *Dietzia* sp. D5, *D. natronolimnaios* which is also isolated from a soda lake water sample [29], and *D. psychrocaliphila* which is isolated from a drain of fish product-processing plant [30], are able to grow at high pH and metabolise hydrocarbons.

Genome sequencing and oxygenase identification

The shotgun sequencing yielded a draft genome sequence consisting of 826 contigs with sizes ranging from 1 to 50 kb. The total number of nucleotides in these contigs was 3,581,770. So far, in the draft genome sequence 43 oxygenases have been identified, of which 28 are monooxygenases and 15 dioxygenases (Figure 2a). A comparison between the available *Dietzia* spp. genomes revealed that the protein sequences of *Dietzia* sp. D5 oxygenases share very high identity to the respective gene sequences in *D. cinnamea* P4 genome. However, as shown in Figure 2a, there are some genes that are specific to each *Dietzia* strains. This may be due to the incompleteness of the genome sequences or to an inherent difference at genome level.

The oxygenase diversity of *Dietzia* sp. D5 was compared to that of *D. cinnamea* and *D. alimentaria* (Figure 2a); the latter with known complete genome sequence [20]. Over 70 and 60% of the monooxygenases and dioxygenases, respectively, identified in genome sequences of the three *Dietzia* spp., exhibited sequence identity higher than 70%. About 14, 13 and 17% of the identified monooxygenases were found exclusively in the genomes of *Dietzia* sp. D5, *Dietzia cinnamea* and *Dietzia alimentaria*, respectively. However, the percentage of the exclusive dioxygenases is relatively higher and accounts for more than 24% of the identified sequences in the genome of each *Dietzia* strain. A summary of the *Dietzia* sp. D5 putative oxygenases is given in Table 1.

The number of oxygenases (both monooxygenases and dioxygenases) encoded by the genomes of *E. coli*, *Bacillus subtilis*, *Rhodococcus jostii* and *Mycobacterium tuberculosis* was also compared to that of *Dietzia* sp. D5 as well as to the other two *Dietzia* genomes; the first two organisms served as a negative control being not rich in oxygenases, whereas the latter two are considered because of their rich oxygenase diversity. *R. jostii* exhibited the highest number of oxygenases while *E. coli* the least. Since the genome sizes of these organisms are different the oxygenase content was normalised by presenting the number per megabases (Mb) of the DNA sequence (Figure 2b). The frequency of encountering oxygenase among genome sequences of the *Dietzia* spp. selected in this study was quite close and was higher than that in *B. subtilis* and *E. coli* genome sequences. The number of monooxygenases found per Mb of the genomic DNA of the three *Dietzia* strains and *M. tuberculosis* is comparable while the dioxygenase content of the *Dietzia* strains is higher than that of *M. tuberculosis*.

Dietzia sp. D5 BVMOs: sequence alignment, conserved motifs, clusters and their analysis

Analysis of *Dietzia* sp. D5 oxygenases has shown that four of the monooxygenases are BVMOs, and for convenience are named hereafter as BVMO1, BVMO2, BVMO3 and BVMO4. BVMO1, BVMO2 and BVMO3 are also identified in *D. cinnamea*

(with 96-97% sequence identity) and *D. alimentaria* (with 73-82% identity), while BVMO4 is unique to *Dietzia* sp. D5.

The amino acid sequences of BVMO1, BVMO2, BVMO3 and BVMO4 were aligned with sequences of other 56 known BVMOs. The aligned sequences were clustered into 7 groups on the basis of sequence similarity, which also reflects the substrate preferences of the BVMOs; each cluster is hence named after the best characterised enzyme in the group - phenylacetone monooxygenase (PAMO), cyclohexanone monooxygenase (CHMO), cyclopentanone monooxygenase (CPMO), cyclopentadecanone monooxygenase (CPDMO), ethionamide monooxygenase (EthA) and hydroxyacetophenone monooxygenase (HAPMO) groups (Figure 3). BVMOs in one of the clusters, named as Group VII BVMOs, are however not well characterised and hence their substrate preference has not yet been clearly established. The BVMOs of *Dietzia* sp. D5 are distributed in three clusters. BVMO3 and BVMO4 are members of the EthA monooxygenase like and CPDMO like clusters, respectively, whereas BVMO1 and BVMO2 clustered in Group VII. BVMO1, BVMO2, BVMO3 and BVMO4 have shown sequence identity of 37-57%, 39-54%, 38-42% and 41-44%, respectively, to members of the respective groups.

The alignment of BVMO sequences highlighted the presence of strictly conserved single site residues and motifs. As shown in Table 2, seven single residues are highly conserved and other 24 residues are also absolutely conserved in six motifs. Among the six motifs, four have been known earlier [31-32] and the remaining two are highlighted for the first time in this study. The position of the conserved residues and motifs are shown using the *Rhodococcus* sp. HI-31 CHMO structure (PDB 3GWD) (Figure 4), in addition representative sequences from each BVMO group were aligned and the conserved motifs are indicated (Figure 5).

BVMOs are known to have two Rossmann motifs: the FAD interacting one (GXGXXG), close to the N-terminal, and the one interacting with NADPH (GXGXX[G/A/S]), located in the middle of the protein sequence. Moreover, two motifs conserved in BVMOs are GXWXXXXYPGXXXD, located 17 to 19 residues after the FAD Rossmann motif, with the aspartic acid that is involved in stabilising and orienting the catalytic arginine [31], and PXXXGXXXFXGXXXHXXXW that precedes the NADPH Rossmann fold by few amino acids and is involved in conformational changes of the enzyme during the catalytic cycles [33].

The newly highlighted motifs DX[I/L][V/I]XXTG and PN(X)₄₋₅G (although the G is not conserved within CPDMO group) are located in the second half of the protein sequence. The DX[I/L][V/I]XXTG motif is close to the NADPH cofactor and it is a so called ATG motif, common to several NAD(P)H dependent flavin binding enzymes [34]. Instead, PN(X)₄₋₅G is far from the active site, it is conserved among BVMOs but it is not present conserved among other closely related enzymes, such as flavin monooxygenases (FMOs).

In addition to the total BVMO sequence analysis, each cluster was also separately analysed to identify potential cluster-unique fingerprint sequence tags. The identified sequence stretches that seem uniquely characterise each cluster are given in Table 3.

One important parameter to be considered is BVMOs' substrate scope. Here, an attempt has been made to summarise the available literature information on substrates (Table 4). Six different groups of substrates which are often used in studies of BVMOs, including alicyclic ketones, substituted alicyclic ketones, aliphatic ketones, aliphatic ketones with an aromatic group, bi- and tri-cyclic ketones and sulfides have been considered, and the reported conversion efficiency from literature is used to categorise the substrate preference of the BVMO groups as "Poor" (for substrate conversion below 5%), "Average" (5-50% conversion) and "Good" (conversion above 50%). EthA and HAPMO groups exhibit relatively higher substrate specificity while BVMOs belonging to CPDMO and PAMO clusters show higher substrate promiscuity compared to the other groups.

Heterologous expression of BVMOs and biotransformations

The putative BVMO genes were cloned and expressed in *E. coli* strain, ArcticExpress(DE3)-RP. This strain co-expresses two cold induced chaperones that have a molecular weight of 57 and 10 kDa (Figure 6). The biotransformation studies using whole cells revealed that BVMO1, BVMO3 and BVMO4 are active while BVMO2 did not show any activity on all substrates tried (Table 5). The conversion achieved by BVMO1 and BVMO3 was smaller than that of BVMO4 which reached about 42% when phenylacetone was used as substrate.

Discussion

Oxygenases are important enzymes for the oxidative synthesis of compounds that are not accessible by chemical routes. However, large scale application of these enzymes is often hindered due to the lack of robust and ideal enzymes, and challenging process requirements [35]. Engineering of existing enzymes and isolation and characterization of new oxygenases have started to yield more robust and versatile enzymes [36-37]. Metagenome and genome sequencing approaches also provide access to novel oxygenase sequences and broaden the available oxygenases portfolio [38-40]. Thus, the sequencing of *Dietzia* sp. D5 genome, a bacterium taxonomically close to oxygenase-rich group of organisms such as *R. jostii* and *M. tuberculosis* is expected to contribute to the growing pool of oxygenases. Indeed, the identification of over 40 oxygenases in the *Dietzia* strains proves that potential.

In addition, the interest in sequencing the genome of *Dietzia* sp. D5 was to prospect for novel BVMOs. In fact, four BVMOs have been identified from the draft genome of this organism. Several bacteria are known to produce multiple BVMOs [41]; for

instance 23 BVMOs have been identified in the genome of *R. jostii* RHA1 [40] and 6 BVMOs from *M. tuberculosis* H37Rv [39]. The catalytic properties of the BVMOs produced by an organism could be different in their physical and catalytic properties as it has been proven in the case of *R. jostii* BVMOs [31]. The difference in the catalytic properties may allow the organisms to efficiently metabolise different substrates.

Different sequence analysis studies have been performed on BVMOs that have increased the understanding of these enzymes [31-32]. Earlier studies revealed the presence of two Rossmann folds and two so-called BVMO motifs, GXWXXXXYPGXXXD and PXXXGXXXFXGXXXHXXXW, which are also identified in this study (Table 2). However, as shown in Table 2, there are some differences between the motif sequences found in this study and the previously published works [31-32]. This may be due to the larger number of sequences considered in the current study. In addition to the four motifs, two new motifs, DX[I/L][V/I]XXTG and PN(X)₄₋₅G, are also reported in this study. The first one was expected to be involved in NADPH binding and it is widespread in several flavoproteins. The residues before TG is in most cases an A, though G and S are also present within the analysed BVMOs. The other conserved motif is PN(X)₄₋₅G. In the group of BVMOs we analysed only BVMO4 has only four amino acids that separate the asparagines from the glycine, nevertheless when a wider sample of sequences it is considered, both four and five residues between PN and G are possible. It is not located close to the active site (Figure 4) and because of its location it might be speculated it has a structural role.

Analysis of the 60 BVMO sequences revealed that there are five fully conserved residues and two residues with conservation higher than 90% (Figure 5, Table 2). Except Arg327, which has catalytic function, and Arg207, which stabilises the cofactor NADPH within the cofactor cleft, the roles of the other residues are not yet known. Mutational analysis of these residues is expected to add to our understanding of these enzymes. Surprisingly, glycine accounts for more than half of the total conserved residues. This could be due to the need for flexibility of these enzymes during cofactor and substrate binding and catalysis. On the other hand, the high flexibility might render BVMOs poor stability.

Several studies have categorised BVMOs into groups based on sequence similarity [31, 40, 42]. Although the clustering tendency is similar in all these studies, the phylogenetic tree branching pattern and the presence or absence of groups vary depending on the diversity of sequences and the type of algorithm used in the analysis. In this study, the 60 sequences considered clustered into seven groups and the BVMOs of *Dietzia* sp. D5 appeared in three clusters: Group VII, EthA monooxygenase like and CPDMO like clusters. Since the catalytic properties of BVMOs are known to vary from cluster to cluster, the distribution of *Dietzia* sp. D5

BVMOs in three clusters suggests that the organism is evolved to efficiently oxidise different substrates. BVMO1 and BVMO2 belong to Group VII BVMOs (Figure 3). Although these two enzymes are from the same organism and belong to one cluster, their sequence identity was only 45%, which may suggest different catalytic properties among the two. The BVMOs belonging to group VII are poorly characterised with respect to substrate specificity, however, based on the little information available, it seems that members of this group preferably oxidise aliphatic and bi-cyclic ketones (Table 4).

The sequence of BVMO3 differs from the rest of the cluster members, and as can be seen in Figure 3 it branched out relatively early. Its identity to the sequences of the BVMOs in the cluster is less than 43%. Moreover, residue W122, which is one of the strictly conserved residues in the other 59 BVMOs considered in this study, is different only in BVMO3. Among the enzymes in this cluster there is EthA monooxygenase, an enzyme from *M. tuberculosis* that is known to catalyse the activation of the pro-drug ethionamide [43], moreover the BVMOs in the EthA cluster effectively oxidise sulfide containing substrates and aliphatic ketones, but unlike BVMOs in other clusters, they poorly oxidise bi- and tri-cyclic ketones.

As in BVMO3, BVMO4 has also diverged from the rest of the CPDMO members fairly early in the tree (Figure 3), indicating a possible difference in the catalytic properties of this enzyme from previously studied BVMOs of the same group. CPDMO like BVMOs are proven to be effective in the biotransformation of large and small cyclic ketones to lactones as well as steroids with high chemo-, regio-, and enantio-selectivity [42, 44-45].

HAPMO like BVMOs have been characterised and show higher affinity towards aliphatic ketones containing an aromatic moiety. Several enzymes belonging to PAMO group have been deeply investigated, mainly due to PAMOs exceptional thermostability [36]. Although PAMO was claimed to be an enzyme with limited substrate scope [46], according to our analysis the BVMOs belonging to this group have quite good substrate promiscuity. Enzymes belonging to the group of CPMO have higher affinity toward alicyclic ketone with substitutions and with more complicated structures, like bicyclic and tricyclic ketones. Finally, the most studied group is the one containing CHMO from *Acinetobacter* sp. NCIMB9871 that was the first BVMO recombinantly expressed and applied since many years for biocatalytic applications [47]. The best substrates for these enzymes are very similar to those of CPMO group.

Although sequence analysis is a powerful tool to identify specific group of enzymes, so far, there is no fingerprint sequence related to the various BVMO clusters. In this study an attempt has been made to identify fingerprint sequences for each BVMO group (Table 3). These sequences can be used as characteristic features in selecting BVMOs with desirable properties and hence will potentially allow using the growing

genomic and metagenomic sequence information in databases. Moreover, these fingerprint sequences can be used to design specific primers to selectively identify genes encoding target BVMOs from genome and metagenome samples.

The expression of the BVMOs identified in the genome of *Dietzia* and the observed biotransformation activities on different substrates supports the idea that the identified sequences are encoding BVMOs. The only sequence that did not show activity was BVMO2 which was expressed as insoluble form (inclusion bodies). Moreover, the bioconversions reported in this work are preliminary and optimization of the BVMOs expression and the biotransformation reactions are needed, which is expected to increase the biotransformation efficiency.

Conclusion

The findings of this study add genus *Dietzia* to the small group of organisms that can serve as a rich source of oxygenases, which can be used potentially to catalyse various oxidation reactions. The four BVMOs identified in the draft genome sequence of *Dietzia* sp. D5 have shown differences within their respective group member sequences and hence are interesting for further studies.

Since the experimental details vary between reports (i.e. different substrates considered, different reaction conditions) and only few enzymes are well characterised in terms of substrate preference it is very difficult to establish an assertive substrate preference profile for each group of BVMOs. Nevertheless, we have tried to utilise all the available information to correlate the BVMO sequences to their biocatalytic properties. Mutational analysis of the conserved residues and structural studies on the newly identified motifs is expected to improve our understanding of this interesting group of enzymes.

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Conflict of interest

The authors have no conflict of interest

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Table 1: Types of oxygenases identified in the genome sequence of *Dietzia* sp. D5

| Type of oxygenase | Number of monooxygenases | Number of dioxygenases |
|---|--------------------------|------------------------|
| Flavin dependent | 20 | 4 |
| Non-heme iron dependent | 0 | 10 |
| Heme-dependent | 5 | 0 |
| Rieske non-heme iron dependent | 1 | 0 |
| Flavin- and heme- dependent | 1 | 1 |
| Di iron- and [Fe(SCys) ₄]-dependent | 1 | 0 |

Table 2: Residues and motifs that are conserved by over 90% of the BVMO sequences considered in the alignment

| Motifs identified | | Position according to amino acid sequence of CHMO from <i>Acinetobacter</i> sp. NCIMB9871 | Description |
|--|---------------------------|--|------------------------------|
| In this study | Previous studies | | |
| GXGXXG | GXGXXG | 13 -18 | First Rossmann [32] |
| GXWXXXXYPGXXXD | A/GGGXWXXX[F/Y]PGMXX D | 43 – 57 | BVMO motif [31] |
| PXXXGXXXFXGXXXHXXX W | FXGXXXHXXXW[P/D] | 150 – 168 | BVMO motif [32] |
| GXGXX[G/A/S] | GXGXX[G/A] | 180 – 185 | Second Rossmann [32] |
| DX[I/L][V/I]XXTG | - | 372 – 379 | ATG motif [34] |
| PN(X) ₄₋₅ G (G is not conserved in CPDMO group) | - | 420 – 427 | PN(X) ₄₋₅ G motif |
| Conserved residues which are not part of the motifs | | | |
| W (except BVMO3 of <i>Dietzia</i> sp. D5) | | 122 | |
| G | | 142 | |
| R | | 207 | NADPH interaction |
| P (A in CDMO) | | 320 | |
| R | | 327 | Catalytic residue |
| G | | 394 | |
| G | | 413 | |

Table 3: Identified fingerprint sequences specific for each BVMO group

| BVMO groups | Motifs unique to the group | Position | Position numbers are according to: |
|-------------------|------------------------------|-----------|--|
| EthA like | PQXWPEDL | 164 – 171 | EthA (NP_218371.1) |
| | TMLQR | 203 – 207 | |
| | PWDXRLCXXP | 288 – 297 | |
| | TATGL | 340 – 344 | |
| HAPMO like | DRPDL | 422 – 426 | HAPMO <i>P. fluorescens</i> (Q93TJ5.1) |
| | SEXXXXYIV | 553- 361 | |
| CPDMO like | DYXXTGXXXXG | 232 – 242 | CPDMO (BAE93346.1) |
| | VDXXVXDXXTAXXLK | 364 – 378 | |
| | CKRPXF | 385 – 390 | |
| | CPTXXXN | 551-557 | |
| PAMO like | DAXTGL | 390 – 396 | PAMO (YP_289549.1) |
| CPMO like | No unanimous motif was found | - | - |
| Group VII | CKR | 293 – 295 | BVMO1 (KF748499) |
| | GCXS[W/Y][Y/F] | 454 – 459 | |
| CHMO like | GSTGXQ | 181 – 190 | CHMO <i>Acinetobacter</i> sp. NCIM9871 (BAA86293.1) |
| | AFGXXEST | 245 – 252 | |
| | FXXMFXXF | 277 – 284 | |
| | ANXXAXXFXXXK | 293 – 304 | |
| | YAKRPLC | 325 – 330 | |
| | DAXXGXYYR | 381 – 389 | |
| | GXFXNXP | 430 – 439 | |
| | SWIFGXN | 489 – 495 | |

Table 4: Summary of the substrate preferences of the different BVMO groups (based on literature information). Poor, Average and Good refer to the conversion of a group of substrates. Variable indicates that for one or more substrates there is a disagreement within the cluster. * = some substrates constitute an exception; ~ = few data are available; NR = not reported.

| Sequence name | Alicyclic ketones | Substituted alicyclic ketones | Aliphatic ketones | Aliphatic ketones with an aromatic group | Bi-tricyclic ketones | Sulfides |
|--------------------|-------------------|-------------------------------|-------------------|--|----------------------|------------|
| EthA group | Poor | Average ~ | Variable | Variable ~ | Poor | Good |
| HAPMO group | Poor | Poor ~ | Poor | Good * | Variable ~ | Poor |
| CPDMO group | Average ~ | Good * | NR | Good ~ | Good * | Good ~ |
| PAMO group | Average * | Good * | Variable | Good * | Good * | Good |
| CPMO group | Good | Good * | Poor | Average ~ | Good * | Variable |
| Group VII | Poor ~ | NR | Good * | NR | Good ~ | Average ~ |
| CHMO group | Good * | Good * | Poor | Average ~ | Good * | Variable ~ |

Table 5: Substrate conversions by the recombinant BVMOs from *Dietzia* sp. D5. “-” no conversion, “+” substrate conversion below 5%, “+ +” more than 5% substrate conversion.

| Recombinant BVMO | Alicyclic ketones | | Aliphatic ketones | | Aliphatic ketones with an aromatic group | Bi-tricyclic ketones |
|------------------|-------------------|--------------------|-------------------|-----------------|--|----------------------|
| | cyclohexanone | cyclopentadecanone | 2-heptanone | 2-pentadecanone | phenylacetone | bicycloheptanone |
| BVMO1 | + | - | + | - | + | - |
| BVMO2 | - | - | - | - | - | - |
| BVMO3 | - | - | - | - | + | - |
| BVMO4 | ++ | + | + | - | ++ | ++ |

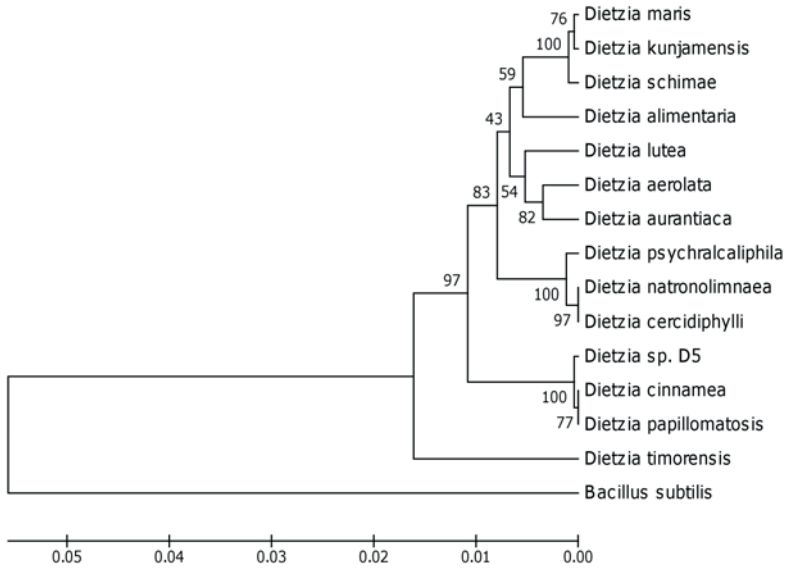
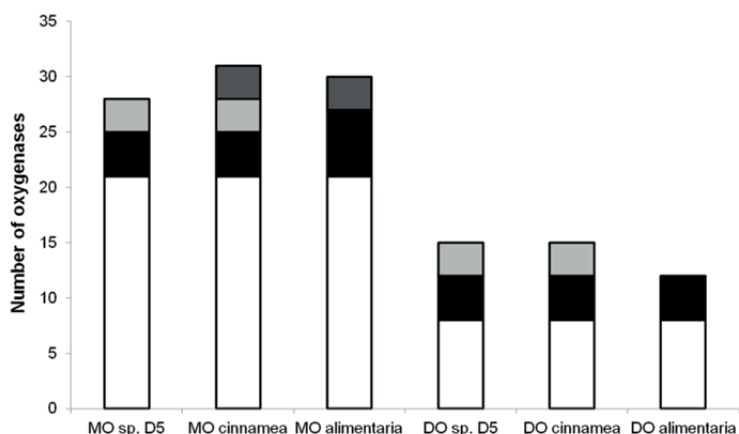


Figure 1: Phylogenetic tree constructed based on the 16S rRNA gene sequence

a



b

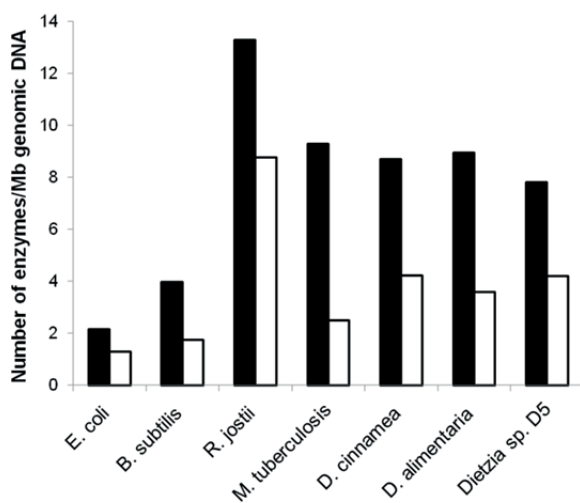


Figure 2: a) Identified potential oxygenase encoding genes in the genome of three *Dietzia* spp. The white, gray and dark gray parts of the bars stand for the number of potential oxygenases in common among the three species, identified only in *Dietzia* sp. D5 and *D. cinnamea* P4, and those that are shared only by *D. cinnamea* P4 and *D. alimentaria* T72, respectively. The black bar represents the oxygenases that were found exclusively in one of the three strains. b) Comparison of oxygenase content between *Dietzia* spp., *Mycobacterium tuberculosis* H37Rv and *Rhodococcus jostii* RHA1 (oxygenase rich genomes), *E. coli* K-12 and *Bacillus subtilis* (negative control). Monoxygenases are represented by the black bars, and dioxygenases by the white bars.

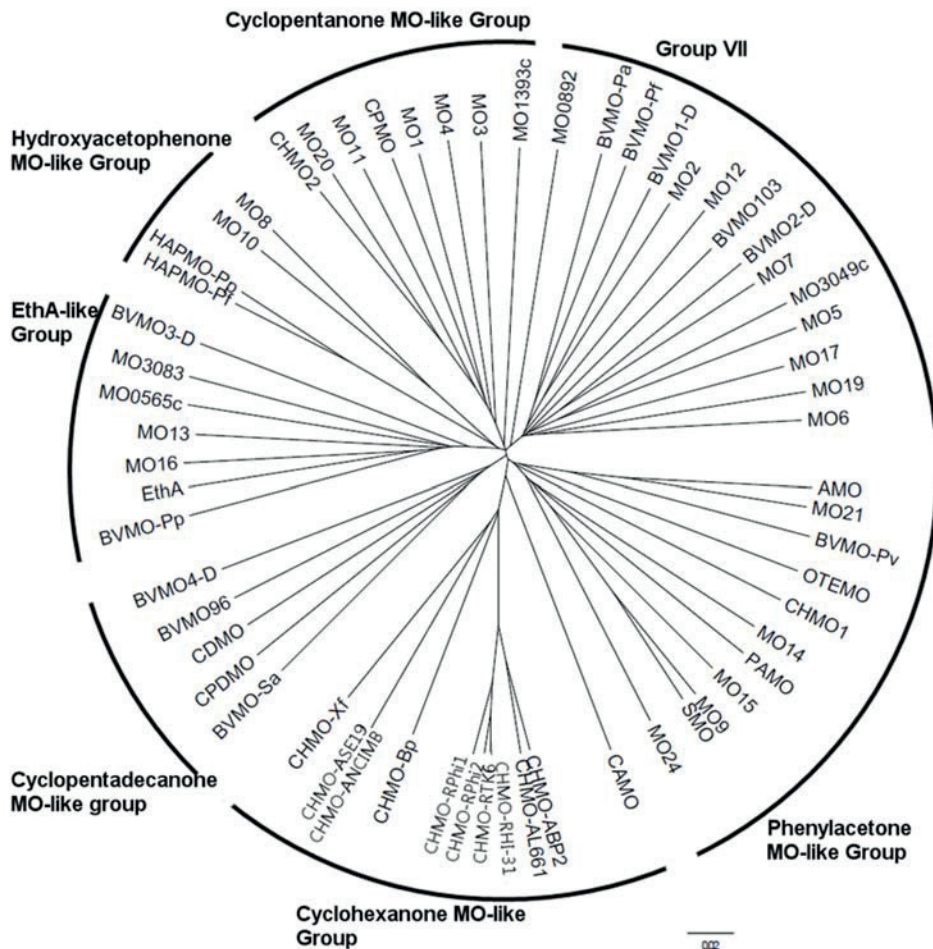
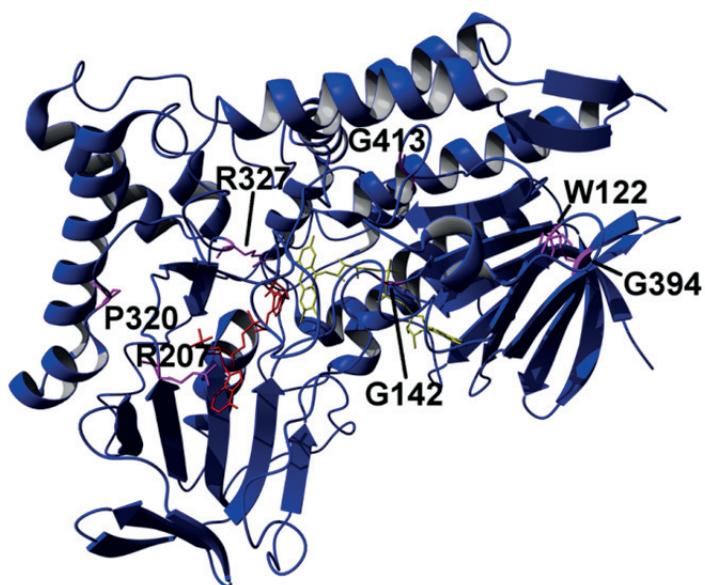


Figure 3: BMO clusters identified on the basis of sequence similarities. *Dietzia* sp. D5 BVMOs are named BVMO1-D, BVMO2-D, BVMO3-D and BVMO4-D. The other acronyms stand for: MO3083 *Mycobacterium tuberculosis* H37Rv (NP_217599.1); MO0565c *Mycobacterium tuberculosis* H37Rv (NP_215079.1); MO13 *Rhodococcus jostii* RHA1 (YP_703731.1); MO16 *Rhodococcus jostii* RHA1 (YP_702882.1); EthA is EthA3854c *Mycobacterium tuberculosis* H37Rv (NP_218371.1); BVMO-Pp is BVMO *Pseudomonas putida* KT2440 (NP_744949.1); BVMO96 *Streptomyces coelicolor* A3(2) (NP_624628.1); CDMO *Rhodococcus ruber* SC1 (AAL14233.1); CPDMO *Pseudomonas* sp. HI-70 (BAE93346.1); BVMO-Sa is BVMO *Streptomyces avermitilis* MA-4680; CHMO-Xf CHMO *Xanthobacter flavus* ZL5 (CAD10801.1); CHMO-ASE19 is CHMO *Acinetobacter* sp. SE19 (AAG10021.1); CHMO-ANCIMB is CHMO *Acinetobacter* sp. NCIMB9871 (BAA86293.1); CHMO-Bp is CHMO *Brachymonas petroleovorans* (AAR99068.1); CHMO-RPhi1 is CHMO *Rhodococcus* sp. Phi1 (AAN37494.1); CHMO-RPhi2 is CHMO *Rhodococcus* sp. Phi2

(AAN37491.1); CHMO-RTK6 is CHMO *Rhodococcus* sp. TK6 (AAR27824.1); CHMO-RHI-31 is CHMO *Rhodococcus* sp. HI-31 (3UCL_A); CHMO-AL661 is CHMO *Arthrobacter* sp. L661(ABQ10653.1); CHMO-ABP2 is CHMO *Arthrobacter* sp. BP2 (AAN37479.1); CAMO *Ilyonectria radicola* (AET80001.1); MO24 *Rhodococcus jostii* RHA1 (YP_705262.1); SMO *Rhodococcus rhodochrous* (BAF48129.1); MO9 *Rhodococcus jostii* RHA1 (YP_708237.1); MO15 *Rhodococcus jostii* RHA1 (YP_702455.1); PAMO *Thermobifida fusca* YX (YP_289549.1); MO14 *Rhodococcus jostii* RHA1 (YP_703398.1); CHMO1 *Brevibacterium* sp. HCU (AAG01289.1); OTEMO *Pseudomonas putida* (3UOV_A); BVMO-Pv is BVMO *Pseudomonas veronii* (ABI15711.1); MO21 *Rhodococcus jostii* RHA1 (YP_708538.1); AMO *Gordonia* sp. TY-5 (BAF43791.1); MO6 *Rhodococcus jostii* RHA1 (YP_701843.1); MO19 *Rhodococcus jostii* RHA1 (YP_705460.1); MO17 *Rhodococcus jostii* RHA1 (YP_705167.1); MO5 *Rhodococcus jostii* RHA1 (YP_702074.1); MO3049c *Mycobacterium tuberculosis* H37Rv (NP_217565.1); MO7 *Rhodococcus jostii* RHA1 (YP_705943.1); BVMO103 *Streptomyces coelicolor* A3(2) (NP_627388.1); MO12 *Rhodococcus jostii* RHA1 (YP_707034.1); MO2 *Rhodococcus jostii* RHA1 (YP_704253.1); BVMO-Pf is BVMO *Pseudomonas fluorescens* DSM50106 (AAC36351.2); BVMO-Pa is BVMO *Pseudomonas aeruginosa* PAO1 (NP_250229.1); MO0892 *Mycobacterium tuberculosis* H37Rv (NP_215407.1); MO1393c *Mycobacterium tuberculosis* H37Rv (NP_215909.1); MO3 *Rhodococcus jostii* RHA1 (YP_703208.1); MO4 *Rhodococcus jostii* RHA1 (YP_703024.1); MO1 *Rhodococcus jostii* RHA1 (YP_706610.1); CPMO *Comamonas* sp. NCIMB9872 (Q8GAW0.3); MO11 *Rhodococcus jostii* RHA1 (YP_706629.1); MO20 *Rhodococcus jostii* RHA1 (YP_707342.1); CHMO2 *Brevibacterium* sp. HCU (AAG01290.1); MO8 *Rhodococcus jostii* RHA1 (YP_708200.1 YP_708201.1); MO10 *Rhodococcus jostii* RHA1 (YP_708241.1); HAPMO-Pp is HAPMO *Pseudomonas putida* JD1 (ACJ37423.1); HAPMO-Pf is HAPMO *Pseudomonas fluorescens* ACB (Q93TJ5.1)

a



b

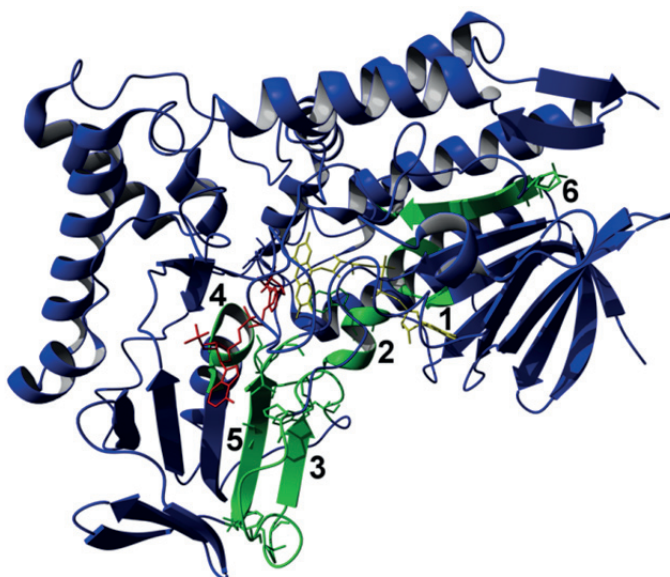


Figure 4: Location of the conserved residues (a) and motifs (b) identified from BVMO sequence alignment study on the structure of CHMO from *Rhodococcus* sp. HI-31.



Figure 5: Alignment of representative BVMOs for each group. Highlighted sequences correspond to: 1) 1st Rossmann Fold; 2) new conserved motif; 3) BVMO motif; 4) 2nd Rossmann Fold; 5) ATG motif 1; 6) PN(X)₄₋₅G motif. Stars highlight the position of the conserved residues.

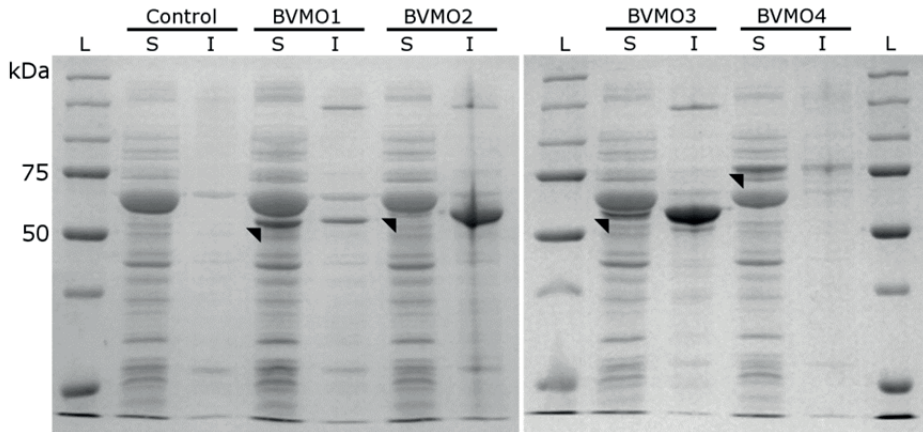


Figure 6: SDS-PAGE presenting the expression in *E. coli* ArcticExpress(DE3)-RP of *Dietzia* sp. D5 BVMOs. L = Ladder AllBlue protein marker (BioRad); S = Soluble crude extract; I = Insoluble protein fraction. Control sample was transformed with pET22b+ without BVMO gene. The expressed protein band is shown by the black arrow in the lanes of soluble fractions. Most of the expressed BVMO2 and BVMO3 are in the insoluble fraction.

Paper II

Cloning and expression of a Baeyer-Villiger monooxygenase oxidising linear aliphatic ketones from *Dietzia* sp. D5

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Abstract

A Baeyer-Villiger monooxygenase has been identified in the genome sequence of *Dietzia* sp. D5. Sequence similarity search revealed that the enzyme belongs to a group of BVMOs that are closely related to ethionamide monooxygenase from *Mycobacterium tuberculosis* (EthA). The BVMO was expressed in *E. coli* BL21-CodonPlus(DE3)-RP and the best expression was achieved when the *E. coli* cells were cultivated in terrific broth (TB) at 15 °C and induced with 0.1 mM of IPTG. Since the purified enzyme did not show any measurable activity, the substrate scope of the BVMO has been determined using whole-cell and crude cell extract systems. The enzyme was most active towards linear aliphatic substrates. However, it has shown a moderate degree of conversion for cyclobutanone, 2-methylcyclohexanone, bicyclo[3.2.0]hept-2-en-6-one, phenylacetone and thioanisole. There was no detectable conversion of ethionamide, cyclohexanone and acetophenone.

Keywords: *Dietzia*; Baeyer-Villiger monooxygenase; aliphatic ketones; ethionamide.

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Abbreviations used: BVMO, Baeyer-Villiger monooxygenase; BVMO3, *Dietzia* sp. D5 BVMO; EthA, Ethionamide monooxygenase; MO0565c and MO3083, *Mycobacterium tuberculosis* BVMOs; MO13 and MO16, *Rhodococcus jostii* BVMOs; BVMO Pp, *Pseudomonas putida* BVMO; BVMO Ar, *Acinetobacter radioresistens* BVMO; BVMO Php, *Physcomitrella patens* BVMO; BVMO Cm, *Cyanidioschyzon merolae* BVMO; BVMO Pf, *Pseudomonas fluorescens* DSM 50106 BVMO; BVMO Pv, *Pseudomonas veronii* MEK700 BVMO; AFL 210, AFL 456 AFL 619 and AFL 838, *Aspergillus flavus* BVMOs; GDH, Glucose dehydrogenase.

Introduction

Baeyer-Villiger monooxygenases (BVMOs) catalyse the oxidation of ketones and heteroatoms (i.e. sulphur, nitrogen, phosphorous, boron and selenium) to lactones and oxides, respectively [1]. BVMOs are considered promiscuous enzymes which oxidise a wide variety of substrates. Among the ketones, small and bulky cyclic ketones, as well as ketones in multicyclic structures and linear aliphatic ketones with and without aromatic substituents can be oxidised by BVMOs. However, each BVMO presents a well defined substrate preference and can oxidise only a limited number of substrates. A systematic investigation and classification of the substrate scope of some BVMOs have been recently reported [2].

BVMOs can also be categorised on the basis of their primary sequence as Type I, Type II and Type O [3]. Among these three groups, the most studied BVMOs are Type I BVMOs which contain FAD as a prosthetic group and are NADPH dependent [4]. Several reports have suggested a further division of Type I BVMOs based on the degree of sequence similarity [5-7]. One of such subdivisions contains ethionamide monooxygenase (EthA) and other enzymes having close sequence similarity with EthA. EthA monooxygenase was isolated from *Mycobacterium tuberculosis* H37Rv and was identified to be responsible for the activation of the pro-drug ethionamide into the bio-active sulfoxide intermediate [8-9]. In addition to ethionamide, the enzyme is active towards aliphatic ketones, such as 2-octanone and 2-decanone, and phenylacetone [9]. Besides EthA monooxygenase, *M. tuberculosis* H37Rv has two other monooxygenases, MO0565c and MO3083, which are known to catalyse the sulfoxidation of thioanisole and MO3083 can also oxidise 2-octanone and bicyclohept-2-en-6-one [10]. Among the 23 BVMOs from *Rhodococcus jostii* RHA1, only two, MO13 and MO16, share significant similarity with EthA monooxygenase. MO16 oxidises different substrates (cyclobutanone, 2-methylcyclopentanone, bicyclohept-2-en-6-one and 2-octanone) but the substrate scope of MO13 is not yet available [5, 7]. A BVMO from *Pseudomonas putida* KT2440 is the other characterised enzyme that shows sequence similarity to EthA monooxygenase; the enzyme oxidises 4-decanone with the highest efficiency [11]. A

recent report shows the presence of another BVMO oxidising ethionamide from *Acinetobacter radioresistens* [12].

Recently, we have sequenced the genome of a strain of *Dietzia*. Four BVMOs have been identified from the draft genome sequence and one of the BVMOs has been characterised and shown to react with sulfides and aldehydes with rare regiospecificity [13-14]. One of the *Dietzia* sp. D5 BVMOs, named BVMO3, is closely related to EthA monooxygenase and in this paper, we report the cloning, expression and substrate scope of this BVMO.

Experimental

Organisms, plasmids and chemicals

E. coli BL21-CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP were purchased from Agilent Technologies (Santa Clara, USA). *E. coli* NovaBlue, *E. coli* BL21(DE3), *E. coli* Rosetta2(DE3) and the plasmid pET-22b(+) were purchased from Novagen (Darmstadt, Germany). QIAGEN Plasmid Mini Kit and QIAEX II Gel Extraction Kit (Qiagen, Sollentuna, Sweden) were used to extract plasmids from cells and DNA from agarose gel, respectively. Genomic DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, USA) from *Dietzia* sp. D5 which was isolated in our laboratory.

All chemicals used in the study are of the highest available purity obtained from standard sources.

Sequence analysis

The multiple sequence alignment analysis of BVMO3 and the other EthA-like monooxygenases was done using the T-coffee algorithm with default parameters and the output figure was prepared using CLC Main Workbench. The multiple sequence alignment was used to derive the phylogenetic tree using Clustal W2 and visualization was obtained using FigTree.

The secondary structures of the BVMOs were predicted using the software CLC Main Workbench.

The sequence of BVMO3 has been deposited in GenBank database under the accession number AHE80562.

Cloning of BVMO3 gene

The gene encoding BVMO3 was amplified from the genomic DNA of *Dietzia* sp. D5 using a pair of primers, BVMO3-F: ATTACCATGGCTGGTAGCACCCACCTC and BVMO3-R: ATTACTCGAGTGATCGGGCCACCTCGTC, which were

designed based on the BVMO3 gene sequence identified in the draft genome sequence. The forward and reverse primers had NcoI and XhoI (underlined) restriction sites, respectively. High Fidelity PCR enzyme mix (Fermentas, Gothenburg, Sweden) was used to amplify the gene following the manufacturer instructions. DMSO (5% *v/v*) was added to the PCR mix to improve the amplification. After purification with Qiagen PCR cleaning kit, the PCR product was digested with NcoI and XhoI and ligated to the expression vector pET-22b(+) digested with the same restriction enzymes. The ligation product was transformed into electrocompetent *E. coli* NovaBlue cells and spread on Luria Bertani (LB) agar plates containing ampicillin. Colonies were picked from the agar plates and recombinant plasmids were extracted and sequenced. The plasmid containing the correct sequence was transformed into the expression hosts *E. coli* BL21(DE3), *E. coli* BL21-CodonPlus(DE3)-RP and *E. coli* ArcticExpress(DE3)-RP.

Protein expression and purification

The recombinant *E. coli* cells were cultivated using low salt LB (tryptone 10 g, yeast extract 5 g, NaCl 5 g per liter), Terrific Broth (TB, composed of tryptone 12 g, yeast extract 24 g, glycerol 4 ml, filtered solution of 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄ 100 ml per liter) and M9 medium (1 M MgSO₄ 2 ml, 20% glucose 20 ml, 1 M CaCl₂ 0.1 ml, M9 salts 200 ml per liter), respectively. The M9 salts comprised Na₂HPO₄·7H₂O 164 g, KH₂PO₄ 15 g, NaCl 2.5 g and NH₄Cl 5 g and water to a final volume of one liter. The media when required contains 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and/or 20 µg/ml gentamycin.

Cultivation of *E. coli* for the recombinant protein expression was initiated by inoculating the culture media with a pre-inoculum corresponding to 5% of the final culture volume of 200 ml in one liter flask. After three hours of cultivation at 30 °C, the OD₆₀₀ was about 0.6 and the protein expression was induced adding IPTG or lactose to final concentration of 0.1 mM and 10 mM respectively. During the expression phase the cells were cultivated at 15 °C for 16 hours with shaking at 150 rpm. The culture was divided into five aliquots of equal volume that were harvested by centrifuging for 10 minutes at 4 °C and 9820 *g* using Sorvall RC5C centrifuge. Then each aliquot was re-suspended in different buffers. The buffers used were: 50 mM sodium phosphate buffer pH 7.5, 100 mM potassium phosphate buffer pH 7.5, 100 mM potassium phosphate buffer pH 7.5 with 0.1% Triton X-100 (*v/v*), 100 mM potassium phosphate buffer pH 7.5 with 10% glycerol (*v/v*) and 100 mM potassium phosphate buffer pH 7.5 with 1 g/L Bovine Serum Albumin (BSA). The cell suspension was placed on ice and lysed in three intermittent cycles of 45 seconds sonication (Hierscher UP400S Ultrasonicator; amplitude 50%, cycle 0.5) with 1 minute break. The sonicated cell suspension was centrifuged for 15 minutes at 4 °C

and 15000 g to remove cell debris and the clear supernatant was used as the enzyme source.

The BVMO was purified from the clear supernatant using immobilised metal ion affinity chromatography on a Ni²⁺ bound column HisTrap™ FF (GE Healthcare, Uppsala Sweden) and eluted with an imidazole gradient from 0 to 300 mM in 100 mM potassium phosphate buffer pH 7.5 containing 200 mM NaCl and 10% glycerol. After protein elution, FAD was added to the fraction containing the purified BVMO3. Finally, desalting and concentration was performed using Vivaspin 20 MWCO 30,000 Dalton centrifugal concentrators (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

The activity of the recombinant enzyme in the crude cell extract and in the purified form was followed by measuring the conversion of 25 mM 2-nonanone by gas chromatography as described below. One enzyme unit is expressed as the amount of enzyme that oxidises one μ mol of 2-nonanone per minute under the standard assay conditions.

Biotransformations using whole cells and crude cell extract

Whole-cell biotransformations were performed using both growing and resting cells. For growing cells, 2 ml TB medium in a 15 ml Falcon tube was inoculated with a pre-culture of *E. coli* BL21-CodonPlus(DE3)-RP cells containing the recombinant plasmid with the BVMO gene. The cells were grown for 3 hours at 30 °C and with shaking at 150 rpm. Immediately after adding IPTG, the biotransformation reaction was initiated by adding the substrate to a final concentration of 5 mM. To boost the regeneration of NADPH, glucose (25 mM) was also added at the moment of induction. Biotransformations were carried out for 16 hours at 15 °C and at a shake rate of 150 rpm. The level of conversion was measured by comparing *E. coli* BL21-CodonPlus(DE3)-RP expressing BVMO3 with cells from the same *E. coli* strain carrying pET-22b(+) without the BVMO3 gene, that served also as a blank for side reactions.

Biotransformation with resting cells was done using a culture grown as described in the section above. At the end of the overnight expression, the cells were harvested by centrifugation and re-suspended in an equal volume of 20 mM sodium phosphate buffer pH 7.4 containing 5 mM substrate and 25 mM glucose and incubated further for 16 hours at 15 °C and 150 rpm.

Biotransformations with crude cell extract were performed by incubating mixtures containing 25 mM glucose, 0.4 U of glucose dehydrogenase (GDH), 133 μ M NADP⁺, 25 mM substrate (from a 1 M stock solution in ethanol) and the crude cell extract for 24 hours at 15 °C and shaking at 150 rpm .

GC analysis

A sample of the biotransformation reaction (400 μ l) was extracted twice with ethyl acetate (500 μ l twice) and analysed using Varian 430-GC gas chromatograph (Agilent Technologies, Santa Clara, USA). Separation of analytes was done using FactorFour VF-1ms 15 m \times 0.25 mm (0.25 μ m) column (Varian, Strathaven, UK) with 1 min hold at 50 $^{\circ}$ C; heating ramp from 50 $^{\circ}$ C to 225 $^{\circ}$ C at a rate of 25 $^{\circ}$ C/min; and 2 min hold at 225 $^{\circ}$ C (the injector and the detector temperature was maintained at 275 $^{\circ}$ C). Identification of the products was done by comparison with retention time of commercial standards.

Results

Sequence analysis

The BVMO3 encoding gene was identified during the draft genome sequence analysis of *Dietzia* sp. D5. The protein sequence similarity search for BVMO3 revealed that it is related to a group of BVMOs containing EthA [9] and other EthA-like BVMOs [5, 10-11]. The phylogenetic relationship of these BVMOs and other well known and characterised BVMOs is shown in Figure 1. The tree illustrates that BVMO3 shares least similarity within the group. The sequence identity between BVMO3 and EthA-like proteins is in the range of 43 and 38% as shown in Table 1 of the Supplementary Information.

The multiple sequence alignment of EthA-like enzymes (Figure 2) shows that residues that are conserved among the other members of the group are not maintained in BVMO3 sequence. Although in some cases the change is simple substitution such as I to L or K to R, there are several cases where BVMO3 residues differ substantially from the conserved amino acids. The substituted amino acids are not part of the conserved cofactor binding motifs but it cannot be speculated if they are active site residues since the structure of these enzymes is not known.

The predicted secondary structures of BVMO3 and the other BVMOs belonging to the EthA-group are compared in Figure 3. The aim of this analysis is to compare the secondary structure similarity of the BVMOs in the group. The comparison shows that the overall secondary structure elements of the different proteins overlap to some extent. The structures are more homogeneous in the N-terminal region in the first part of the protein sequence until the end of the α -helix region 1, instead in the remaining regions of the protein (α -helix region 2 and β -sheet regions 1 and 2) there is no unanimous alignment between the protein considered in the analysis.

Cloning, expression and purification

The BVMO3 gene was cloned in the commercial expression vector pET-22b(+). The gene is 1556 nucleotides long and the calculated mass of the recombinant protein is 61.6 kDa. This calculation takes into account the mass of the leader peptide PelB and the His-tag. Although the gene is expressed with PelB which is assumed to help exporting the protein to the periplasmic region in order to avoid protein aggregation, the expressed protein remained in the cytoplasm and resulted mainly in inclusion bodies (Figure 4).

Transformation of the BVMO3 gene in the commonly used *E. coli* expression host BL21(DE3) did not give any detectable expression (Figure 4). This was presumably caused by the high GC content (>70%) of the gene and the presence of several rare codons. Thus, *E. coli* strains designed to overcome codon bias and high GC content gene expression problems, namely *E. coli* Rosetta2(DE3), *E. coli* BL21-CodonPlus(DE3)-RP and *E. coli* ArcticExpress(DE3)-RP, were chosen. Active BVMO3 was expressed by *E. coli* BL21-CodonPlus(DE3)-RP and *E. coli* ArcticExpress(DE3)-RP which carry a plasmid for the expression of rare tRNAs. The ArcticExpress strain encodes also for cold induced chaperones (one of them having a molecular weight of 60 kDa, which in the SDS-PAGE overlaps the 61.6 kDa protein band of BVMO3) and hence the BVMO expression with ArcticExpress cells had to be performed at 10-12 °C to allow the co-expression of the chaperones. However the low temperature for cultivation resulted in low cell density, even when the expression phase was prolonged to 24 hours. Moreover, *E. coli* BL21-CodonPlus(DE3)-RP have shown higher biotransformation conversions than *E. coli* Arctic-Express(DE3)-RP (data not shown). Therefore, *E. coli* BL21-CodonPlus(DE3)-RP was chosen as production host for further studies.

Optimization of some expression parameters including cultivation temperature, inducer type and concentration, and cultivation media, was carried out in order to increase the expression of soluble BVMO3 (data not shown). The highest amount of soluble enzyme in *E. coli* BL21-CodonPlus(DE3)-RP was obtained when the cells were induced with 0.1 mM IPTG, grown at 15 °C and in TB medium. These conditions were used in all the subsequent studies.

It has been suggested that EthA-like BVMOs could be membrane associated [9, 15], therefore the choice of a proper cell lysis buffer is very important to keep the protein in its active form in the soluble fraction of the crude cell extract. The choice of the buffer salts and the additives was based on reports available from scientific literature [8-9, 13, 16]. Although the different resuspension buffers did not influence greatly the amount of BVMO3 present in the soluble crude cell extract, the SDS-PAGE analysis revealed that the Triton X-100 had slightly increased the amount of soluble BVMO (Figure 1 Supplementary Information). However, the activity of the BVMO3 in potassium phosphate buffer containing glycerol was higher than the ones measured

in the other buffers (Figure 5). Contrary to what was shown in the SDS-PAGE, the activity of the cell extract containing Triton X-100 was lower than the other preparations.

The stability of BVMO3 in the crude cell extract was estimated by measuring the conversion of 2-nonanone after 14 days of storage at 4 °C or at -20 °C (Figure 5). The enzyme activity was strongly reduced after two weeks storage in the refrigerator (4 °C), while it was maintained or decreased slightly when stored at -20 °C. Therefore, potassium phosphate buffer with 10% glycerol was used as cell resuspension buffer for the following experiments.

The recombinant enzyme was purified using metal ion affinity chromatography, but the purified enzyme showed very little activity (<5% conversion) that was completely lost after few days (data not shown).

Biotransformations using growing cells, resting cells and crude cell extract

A range of molecules containing different functional groups (ketones with aliphatic, alicyclic or aromatic moieties and sulphides) that are known to be transformed by other BVMOs were used as substrates (Table 1 and 2). Little to moderate conversion was measured in case of cyclobutanone, 2-methylcyclohexanone, bicyclo[3.2.0]hept-2-en-6-one, phenylacetone and thioanisole by growing recombinant cells, while acetophenone, cyclohexanone and even ethionamide were not converted by BVMO3 (Table 1). On the other hand, excellent conversions were achieved for linear aliphatic ketones, with the highest degree of conversion measured for 2-nonanone and 3-decanone by growing cells (Table 2). Interestingly, when the keto-group is on the second carbon there was only one oxidation product but when it is on the third carbon two products are observed by gas chromatography. The ratios among the two products vary for the different 3-keto substrates (Table 2). Bioconversion of aliphatic ketones was also investigated using the recombinant resting cells and crude cell extract (Table 2). Resting cells were prepared by resuspending the recombinant *E. coli* cells after overnight expression in sodium phosphate buffer. The conversions achieved by the resting cells were lower than that of growing cells or crude cell extract (Table 2).

The reactions prepared with crude cell extract exhibited complete conversion of 5 mM substrate after overnight incubation, hence a higher substrate concentration (25 mM) was used in the conversion studies. The cofactor NADPH was enzymatically regenerated by glucose dehydrogenase and the co-substrate glucose was added in stoichiometric amount to the substrate. The highest substrate conversion by crude cell extract was achieved for 2-nonanone and 2-decanone (Table 2).

When the enzyme was assayed using 2-nonanone as substrate, the activity in the crude cell extract and the growing cells preparations was about 14.3 U/ml and 4.7 U/ml, respectively.

Discussion

Dietzia sp. strains are known to have a variety of oxygenases [17-19]. This work presents cloning, expression and substrate scope of a BVMO isolated from *Dietzia* sp. D5. Sequence analysis of this monooxygenase revealed that it is an EthA monooxygenase-like enzyme and shares high sequence similarity with other characterised BVMOs from *M. tuberculosis* H37Rv [9-10], *P. putida* KT2440 [11] and *R. jostii* RHA1 [5]. There has been a great interest on EthA monooxygenase mainly because of its physiological role of converting the pro-drug ethionamide to the biologically active form. Mutations on this gene and its regulator EthR are the cause of the formation of ethionamide-resistant strains of *M. tuberculosis* [20].

BVMO3 amino acid sequence differs at sites that are highly conserved in the EthA and EthA-like monooxygenases (Figure 2). Further analysis highlighting these differences is given by the prediction of the secondary structure of BVMO3 and the other EthA-like BVMOs, presented in Figure 3. It seems that the secondary structure is not strictly conserved among this group of enzymes. On the N-terminus side, BVMO3 secondary structure resembles that of EthA monooxygenase, MO16 and BVMO Pp. The common pattern is present up to the amino acid residue 200, corresponding to the chunk of sequence containing the two Rossmann folds and the so-called BVMO motif [21]. A similar analysis made for other groups of BVMOs, namely cyclohexanone monooxygenase-like BVMOs and phenylacetone monooxygenase-like BVMOs shows that there is a much higher degree of consensus among the secondary structures and a pattern of α -helix and β -sheets throughout the whole secondary structure alignment can easily be identified (Figure 2 a,b Supplementary Information). This observation suggests that the sequence similarity of EthA-like BVMOs might not correspond to similar protein folding, however to fully further support this statement, determination of crystal structure for one or more enzymes of this type is necessary.

Although members of this BVMO group are expected to have a similar substrate scope, the results from previously published characterizations do not support this assumption (Table 3). Interestingly, BVMO3 did not oxidise ethionamide but catalyses the oxidation of other sulphides, such as thioanisole. The ability to oxidise thioanisole seems to be a common feature among this group of BVMOs and several other BVMOs which do not belong to this group. The oxidation of cyclohexanone and acetophenone by EthA-like BVMOs is very poor, while different levels of conversions are reported for the oxidation of bicyclohept-2-en-6-one, phenylacetone and 2-octanone. BVMO3 exhibited strong preference towards linear aliphatic ketones such as 2-nonanone and 3-decanone; similar behaviour was observed for *P. putida* BVMO and EthA monooxygenase from *M. tuberculosis* but other enzymes of the group (i.e. MO3083 and MO0565c) do not show such a preference (Table 3). The reason why the other enzymes did not convert aliphatic ketones might be related to

their poor expression, such as in the case of MO13 from *Rhodococcus* [7], due to choosing a restricted number of substrates to investigate the reactivity of the BVMOs (i.e. in case of MO3083c and MO0565 the only linear aliphatic ketone tested was 2-octanone) or due to intrinsic properties.

The substrate scope of EthA-like and non EthA-like BVMOs which are able to convert linear aliphatic ketones is compared in Table 3. The non EthA-like BVMOs share less than 20% sequence identity with BVMO3 but can oxidise linear aliphatic ketones with good or moderately good conversion. When substrates other than linear aliphatic ketones are considered, differences in the conversion obtained emerge among these enzymes. Interestingly, AFL 456, AFL 619 and AFL 838 are all unable to oxidise cyclohexanone that is a characteristic property of EthA-like BVMOs. In particular AFL 619 from *Aspergillus flavus* converts approximately the same substrates as BVMO3, despite their low overall sequence similarity. This observation suggests that BVMO's sequence similarity does not always indicate substrate scope similarity and that the experimental characterization of the enzyme is the only tool to determine the substrate preference of BVMOs.

Comparison of the predicted secondary structure of the aliphatic ketone-converting BVMOs with EthA-like BVMOs (Figure 2 c Supplementary Information) shows that, in addition to low sequence identity, there is great heterogeneity in the distribution of the secondary structure motifs along the protein sequence. This observation hints that, despite the low overall sequence similarity and differences in the secondary structure, BVMOs may exhibit similar substrate scope if the active site area is similar. The information about which residues form the active site in BVMO3 or other EthA-like BVMOs is still limited as none of these enzymes has been crystallised and its three dimensional structure determined.

Among the substrates tested, BVMO3 has shown high conversions for linear aliphatic ketones. The maximum conversion of 89.8% and 80.8% was reached for 2-nonanone and 3-decanone, respectively by growing cells (Table 2).

In whole-cell biotransformations, higher conversion yields were obtained with growing cells as compared to the resting cells, even though it was previously reported that resting cells biotransformation resulted in higher conversion [11]. It has of course to be underlined that when using whole-cells for biotransformations such as the *E. coli* strain used, the metabolic efficiency of the cells after expression phase, the efficiency of the cofactor regeneration efficiency or the loss of the cofactors due to cell permeabilization and oxidative stress can affect the oxidation efficiency [22].

In case of the crude cell extract, the amount of substrate used was five-times higher compared to that used for the whole-cell biotransformations. When 5 mM of the linear ketones were added to the crude cell extract the conversion was complete within a short time, possibly because of the higher enzyme load or the reduced mass

transfer limitations due to absence of cell barriers and easy mixing. Aliphatic ketones were confirmed to be the most favoured substrates for BVMO3. The differences between conversion by whole-cell and in crude cell extract systems were negligible when the aliphatic 2-ketones are considered. Nevertheless, the conversion of 3-nonanone and 3-decanone by the crude cell extract was less efficient. Moreover the ratio between the oxidation products varied between biotransformation methods.

There was no detectable conversion of cyclohexanone, acetophenone and ethionamide. Cyclohexanone and acetophenone are expected to be poor substrates for this group of BVMOs (Table 3); however, ethionamide which is a known substrate for EthA [8-9] was not converted by BVMO3. This evidence suggests once again that sequence similarity does not ensure similar substrate scope for this group of enzymes.

Although a satisfactory level of soluble recombinant protein expression was achieved using *E. coli* BL21-CodonPlus(DE3)-RP, this report shows that BVMO expression can be a cumbersome process, especially when the gene is high GC content and contains several rare codons. This could be solved by synthesising the gene of interest, however screening of commercially available *E. coli* strains that overcame the problem could be an alternative. *E. coli* BL21-CodonPlus(DE3)-RP and *E. coli* ArcticExpress(DE3)-RP demonstrated to produce soluble protein. The enzymatic activity measurement indicated that *E. coli* BL21-CodonPlus(DE3)-RP was a better expression host for BVMO3. The protein expression level was further increased by optimising a number of parameters, such as culture media, inducer type and concentration and cultivation temperature. The expression level might be further increased if the expression vector could be changed, for instance a vector with fusion tags that increases protein solubility [23].

An interesting finding of this study is the influence of the resuspension buffer on the recovery of functional enzyme in the crude cell extract. It was already suggested that additives can increase the stability of BVMOs after cell lysis and in particular these additives can be fundamental when the protein interacts with the cell membrane, as it has been speculated for this type of BVMOs [8-9, 15]. The presence of potassium appears to have a slightly beneficial effect. In agreement with previous reports [8-9], Triton X-100 slightly increased the amount of protein in the crude cell extract but it has a negative effect on the activity of BVMO3 (Figure 5). Glycerol proved to be a good stabilizer for the enzyme, especially when the enzyme was stored at -20 °C (Figure 5). Glycerol and other polyols are known to provide stabilising effect through preferential exclusion mechanism [24]. Irrespective of the resuspension buffer used, prolonged storage of BVMO3 at 4 °C of BVMO3 reduced the enzymatic activity, suggesting that this BVMO is rather unstable. In fact, the purified BVMO3 lost all of its activity rapidly. Such loss of activity could be reduced upon understanding the mechanism of inactivation of the enzyme and thereby modifying the purification strategy (i.e. using purification buffers with detergents or hydrophobic molecules,

testing other types of protein chromatography) and enzyme formulation for storage. This would allow further characterization of BVMO3.

Conclusions

BVMO3 from *Dietzia* sp. D5 has been characterised for its substrate scope and linear aliphatic ketones are shown to be the preferred substrates. Although this enzyme shows high sequence similarity with previously reported EthA and EthA-like BVMOs, it was shown that they have differences in conserved amino acids and BVMO3 could not oxidise ethionamide. Furthermore, a comparison with other BVMOs with similar substrate scope suggested that there is no clear correlation between sequence similarity and substrate scope. This opens up the challenge to identify the residues that determine substrate specificity in this group of BVMOs, which could be achieved with the help of X-ray crystallography and systematic mutagenesis studies.

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Table 1: Substrates conversion by BVMO3. Whole-cell biotransformations with growing cells were performed with 5 mM substrate. The reaction mixtures were incubated at 15 °C with shaking at 150 rpm for 16 hours.
 NC: not converted.



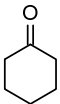
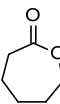
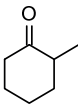
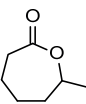
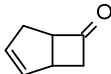
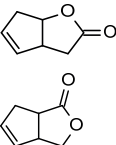
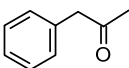
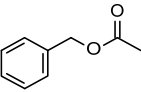

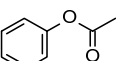
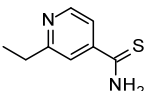
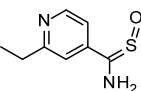


| Substrate | | Products | Growing cells |
|-------------------------------|---|---|---------------|
| Cyclobutanone |  |  | 34.2% |
| Cyclohexanone |  |  | NC |
| 2-Methylcyclohexanone |  |  | 21.0% |
| Bicyclo[3.2.0]hept-2-en-6-one |  |  | 13.5% |
| Phenylacetone |  |  | 35.2% |
| Acetophenone |  |  | NC |
| Ethionamide |  |  | NC |
| Thioanisole |  |  | 32.0% |

Table 2: Conversion of aliphatic ketone substrates by BVMO3. Whole-cell biotransformations with growing and resting cells and crude cell extract were performed with 5 mM substrate, while biotransformations with crude cell extract contained 25 mM substrate and glucose/glucose dehydrogenase for cofactor regeneration. The reaction mixtures were incubated at 15 °C with shaking at 150 rpm for 16 hours (whole cells and resting cells) or 24 hours (crude cell extract). In brackets is the ratio between product a and b, respectively.

NC: not converted.

ND: not determined.

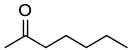
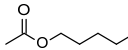
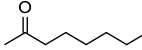
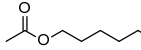
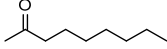
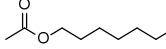
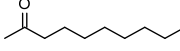
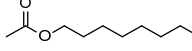
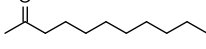
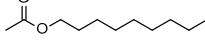
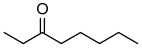
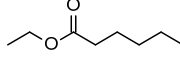
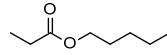
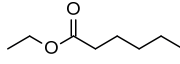
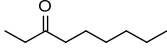
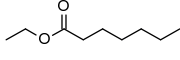
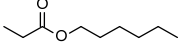
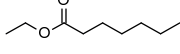
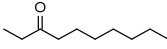
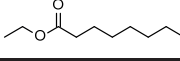
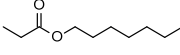
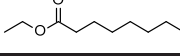
| Substrate | Product | Conversion | | | |
|--------------|--|--|---------------|--------------------|-------|
| | | Growing cells | Resting cells | Crude cell extract | |
| 2-Heptanone |  |  | 41.1% | Traces | 73.6% |
| 2-Octanone |  |  | 60.8% | 6.6% | 78.3% |
| 2-Nonanone |  |  | 89.8% | 3.2% | 82.4% |
| 2-Decanone |  |  | 80.8% | 10.5% | 83.7% |
| 2-Undecanone |  |  | 63.9% | NC | 57.9% |
| 3-Octanone | a  b  |   | 44.3% | 5.9% | 43.6% |
| | | | | | |
| 3-Nonanone | a  b  |   | 64.2% | 1.6% | 45.9% |
| | | | | | |
| 3-Decanone | a  b  |   | 85.9% | Traces | 59.0% |
| | | | | | |

Table 3: Comparison of substrate conversions reported for other EthA-like monooxygenases and other BVMOs converting linear substrates.

Degree of conversion: “+ +” corresponds to more than 55%, “+” is between 55% and 5%, “(+)” is less than 5%, “-” is no conversion and “NA” means that there is no data available. MO13 is not included because it was not reported to convert any substrate so far, recombinant BVMO-Ar was tested only for the oxidation of 4-phenyl-2-butanone and ethionamide.

| | BVMO3 | MO3083 [10] | MO 0565c [10] | MO16 [5] | EthA [9-10] | BVMO _{Pp} [11] | BVMO _{Pp} [25] | BVMO _{Cm} [25] | BVMO _{Pf} [26] | BVMO _{Pv} [16] | AFL 210 [27] | AFL 456 [27] | AFL 619 [27] | AFL 838 [27] |
|------------------------|-------|-------------|---------------|----------|-------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|--------------|--------------|--------------|--------------|
| Cyclobutanone | + | NA | NA | + | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| Cyclohexanone | - | NA | NA | (+) | - | (+) | (+) | ++ | NA | + | + | - | - | - |
| Bicyclohept-2-en-6-one | + | (+) | - | + | (+) | NA | ++ | ++ | NA | NA | ++ | ++ | + | ++ |
| Phenylacetone | + | NA | NA | NA | ++ | - | ++ | ++ | NA | NA | ++ | ++ | + | ++ |
| Acetophenone | - | NA | NA | NA | - | - | NA | NA | NA | + | ++ | + | - | + |
| Thioanisole | + | + | ++ | NA | NA | NA | NA | NA | NA | NA | + | + | (+) | - |
| 2-Octanone | ++ | (+) | - | + | ++ | (+) | ++ | ++ | + | + | ++ | ++ | + | ++ |
| 2-Decanone | ++ | NA | NA | NA | ++ | ++ | NA | NA | ++ | ++ | ++ | ++ | + | ++ |

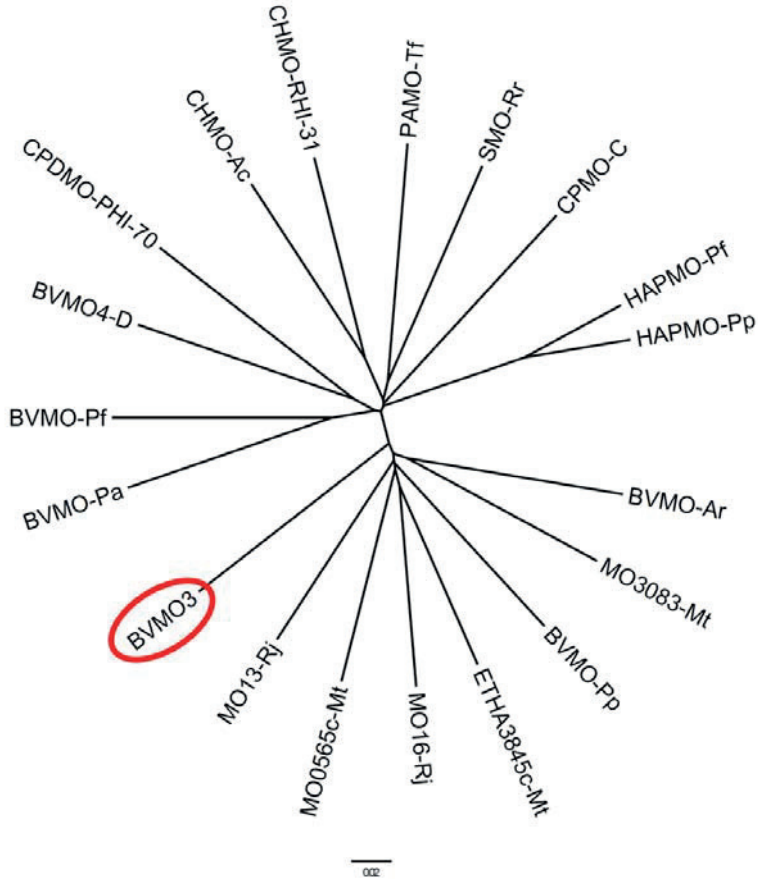


Figure 1: Phylogenetic tree derived from sequence alignment of representative BVMOs and BVMO3 from *Dietzia* sp. D5.

The accession number of each BVMO sequence considered in the alignment is given in bracket. BVMO3 from *Dietzia* sp. D5 (AHE80562.1), MO0565c-Mt from *Mycobacterium tuberculosis* H37Rv (NP_215079.1), ETHA3854c-Mt from *Mycobacterium tuberculosis* H37Rv (NP_218371.1), MO16-Rj from *Rhodococcus jostii* RHA1 (YP_702882.1), BVMO-Pp from *Pseudomonas putida* KT2440 (NP_744949.1), MO13-Mt *Rhodococcus jostii* RHA1 (YP_703731.1), MO3083-Mt from *Mycobacterium tuberculosis* H37Rv (NP_217599.1), BVMO-Ar from *Acinetobacter radioresistens* (ADF32068), BVMO-Pa from *Pseudomonas aeruginosa* PAO1 (NP_250229.1), BVMO-Pf from *Pseudomonas fluorescens* DSM50106 (AAC36351.2), HAPMO-Pf from *Pseudomonas fluorescens* ACB (Q93TJ5.1), HAPMO-Pp from *Pseudomonas putida* JD1 (ACJ37423.1), BVMO4-D from *Dietzia* sp. D5, CPDMO-PHI-70 from *Pseudomonas* sp. HI-70 (BAE93346.1), CHMO-Ac is CHMO *Acinetobacter* sp. NCIMB9871 (BAA86293.1), CHMO-RHI-31 from *Rhodococcus* sp. HI-31 (3UCL_A), PAMO-Tf from *Thermobifida fusca* YX (YP_289549.1), SMO-Rr from *Rhodococcus rhodochrous* (BAF48129.1) and CPMO-C from *Comamonas* sp. NCIMB9872 (Q8GAW0.3).

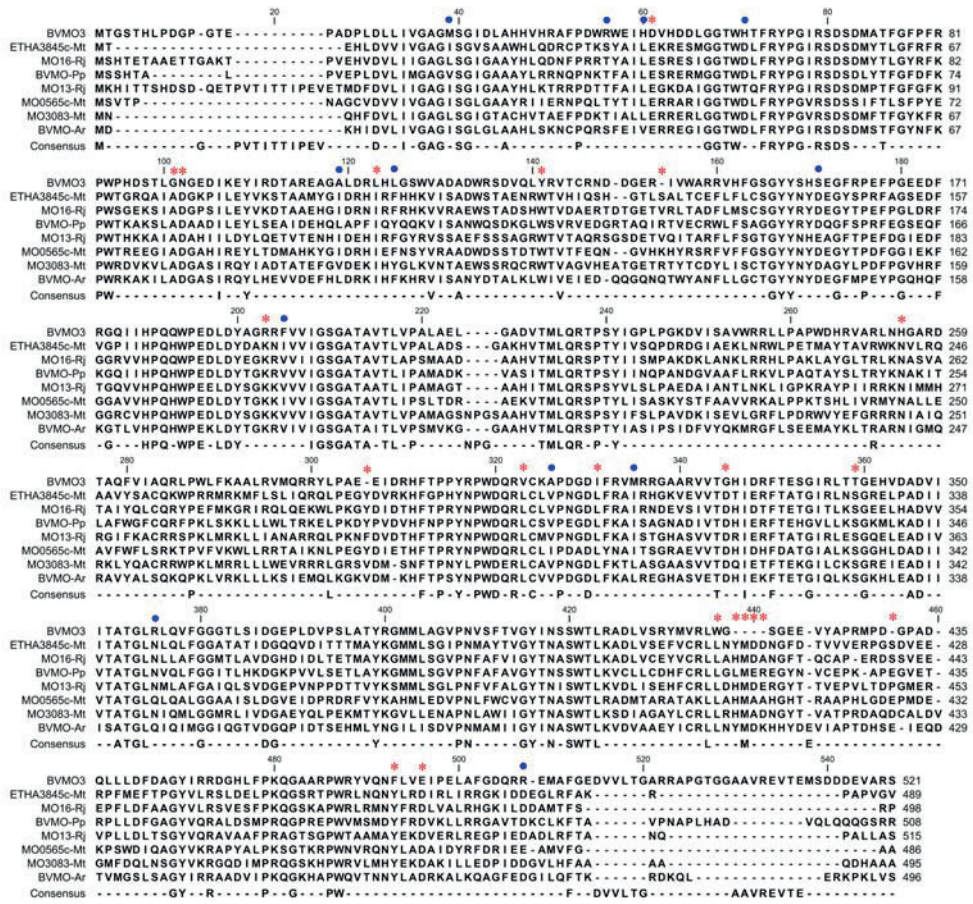


Figure 2: Multiple sequence alignment of EthA-like monoxygenases. Red stars represent the residues that are unique in BVMO3, blue dots highlight the residues with different physicochemical properties exclusive for BVMO3

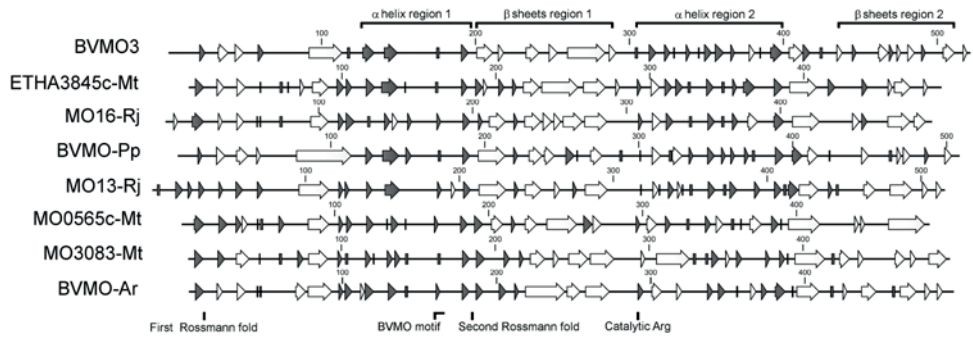


Figure 3: Predicted secondary structure of BVMO3 aligned to the predictions from the other members of EthA group. White arrows represent α -helix and grey arrows represent β -sheets.

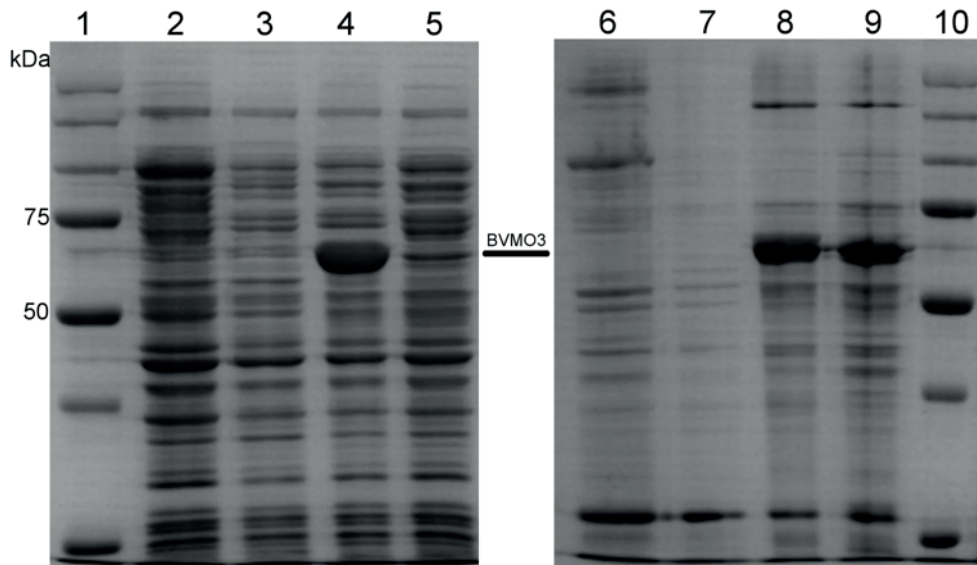


Figure 4: SDS-PAGE comparing the expression of BVMO3 in different *E. coli* strains. The protein concentration of each soluble crude fraction was measured prior to SDS-PAGE and a similar amount of protein was loaded for each *E. coli* strain. Lane 1 and 10: AllBlue Marker; lane 2 and 6: soluble and insoluble crude cell extract fractions from *E. coli* BL21(DE3); lane 3 and 7: soluble and insoluble crude cell extract fractions from *E. coli* Rosetta2(DE3); lane 4 and 8: soluble and insoluble crude cell extract fractions from *E. coli* ArcticExpress(DE3)-RP; lane 5 and 9: soluble and insoluble crude cell extract fractions from *E. coli* BL21-CodonPlus(DE3)-RP.

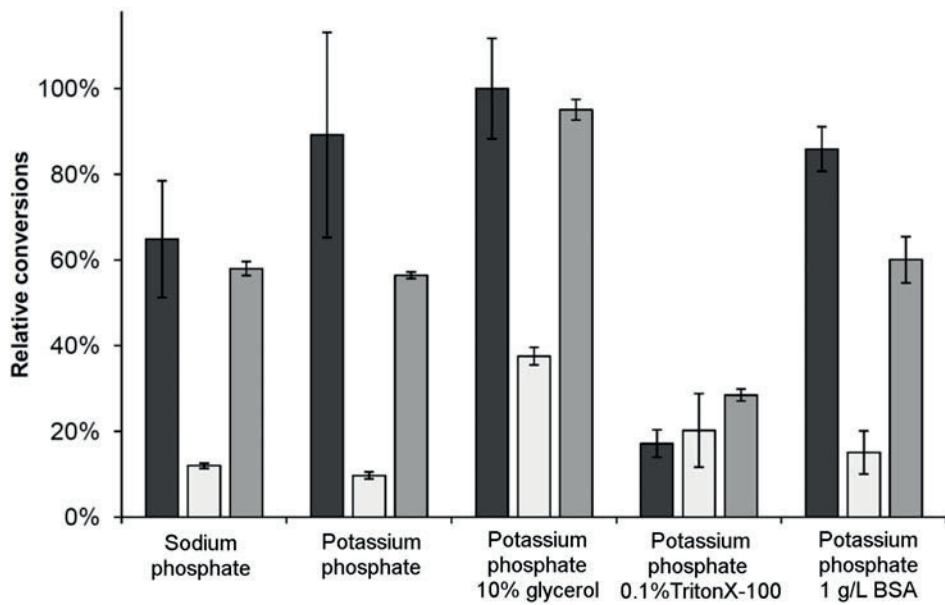


Figure 5: Comparison of the enzymatic activity of BVMO3 in different cell lysis buffers. The enzymatic activity was measured immediately after cell lysis (black bars) and after 14 days when the enzyme was stored at 4 °C (white bars) and -20 °C (grey bars). The experiment was done twice and standard deviations were calculated from the experimental results.

Supplemental information

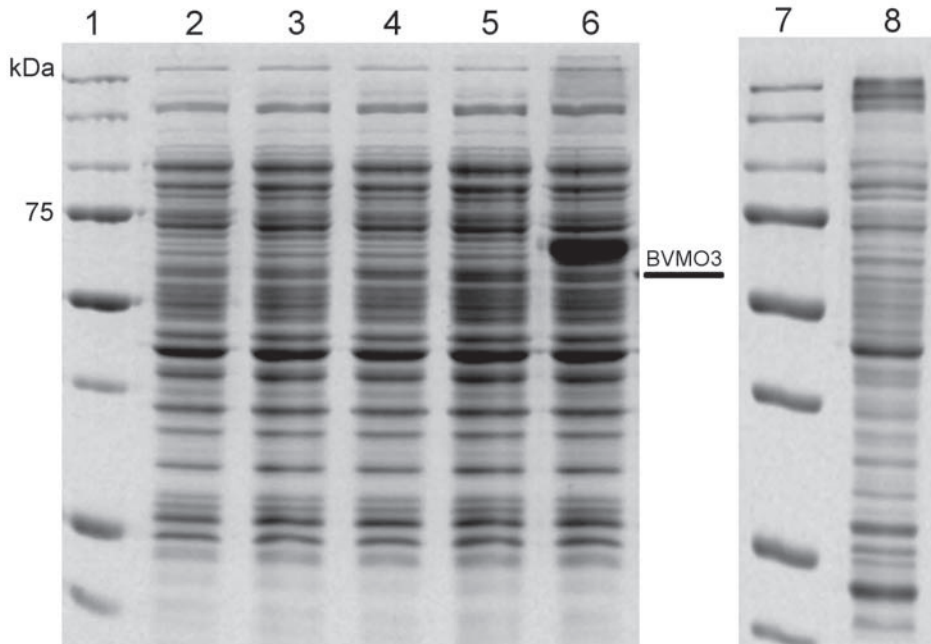
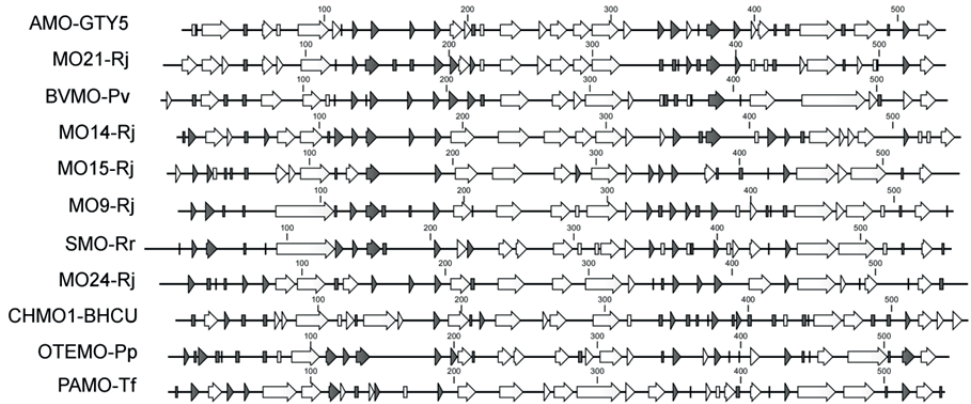
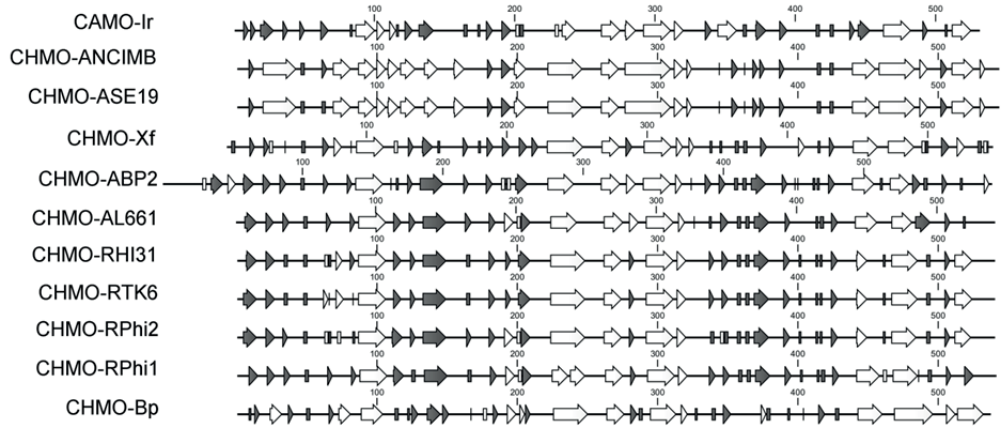


Figure 1: SDS-PAGE showing the influence of resuspension buffers on BVMO3 solubility. The expression host used is *E. coli* BL21 CodonPlus (DE3)-RP. Lane 1 and 7: AllBlue Marker; lane 2: soluble fraction in sodium phosphate buffer; lane 3: soluble fraction in potassium phosphate buffer; lane 4: soluble fraction in potassium phosphate buffer with 10% glycerol; lane 5: soluble fraction in potassium phosphate buffer with 0.1% Triton X-100; lane 6: soluble fraction in potassium phosphate buffer with 1 g/L BSA; lane 8: soluble fraction of *E. coli* expressing pET22b+ without BVMO3 resuspended in sodium phosphate buffer. The band corresponding to BVMO3 is indicated by the horizontal bar and the thick band in lane 6 corresponds to BSA added to the buffer.

a**b**

c

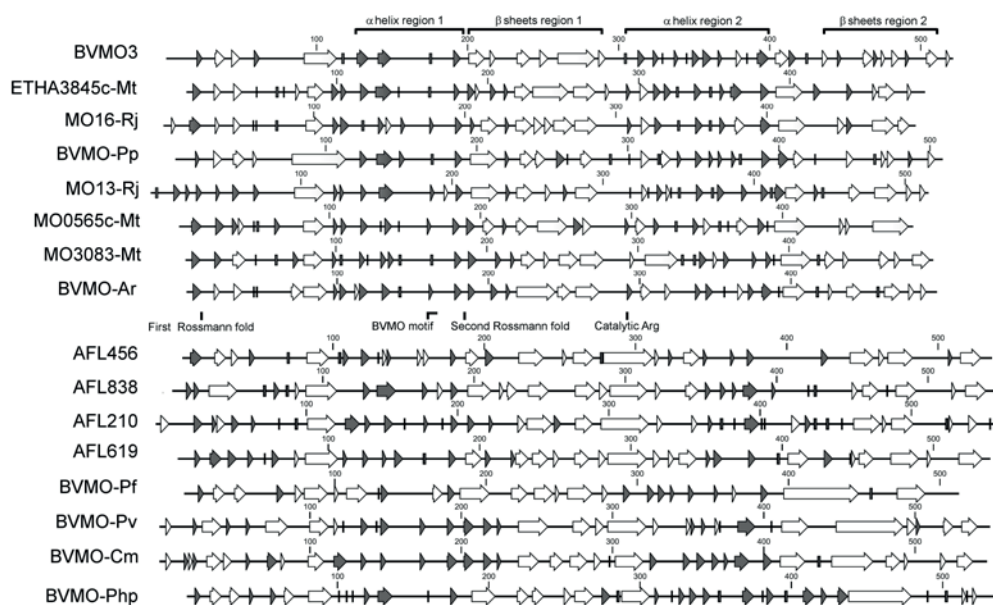


Figure 2: Aligned predicted secondary structure of Phenylacetone monooxygenase (PAMO) and PAMO-like BVMOs (a), Cyclohexanone monooxygenase (CHMO) and CHMO-like BVMOs (b) and EthA-like BVMOs and BVMOs converting linear aliphatic ketones (c). White arrows represent α -helix and grey arrows represent β -sheets.

The acronyms in the figure are: AMO-GTY5, *Gordonia* sp. TY-5 (BAF43791.1); MO21-Rj, *Rhodococcus jostii* RHA1 (YP_708538.1); BVMO-Pv, *Pseudomonas veronii* (ABI15711.1); MO14-Rj, *Rhodococcus jostii* RHA1 (YP_703398.1); MO15-Rj, *Rhodococcus jostii* RHA1 (YP_702455.1); MO9-Rj, *Rhodococcus jostii* RHA1 (YP_708237.1); SMO-Rr, *Rhodococcus rhodochrous* (BAF48129.1); MO24-Rj, *Rhodococcus jostii* RHA1 (YP_705262.1); CHMO1-BHCU, *Brevibacterium* sp. HCU (AAG01289.1); OTEMO-Pp, *Pseudomonas putida* (3UOV_A); PAMO-Tf, *Thermobifida fusca* YX (YP_289549.1); CAMO-Ir, *Ilyonectria radicola* (AET80001.1); CHMO-ANCIMB, *Acinetobacter* sp. NCIMB9871 (BAA86293.1); CHMO-ASE19, *Acinetobacter* sp. SE19 (AAG10021.1); CHMO-Xf, *Xanthobacter flavus* ZL5 (CAD10801.1); CHMO-ABP2, *Arthrobacter* sp. BP2 (AAN37479.1); CHMO-AL661, *Arthrobacter* sp. L661 (ABQ10653.1); CHMO-RHI31, *Rhodococcus* sp. HI-31 (3UCL_A); CHMO-RTK6, CHMO *Rhodococcus* sp. TK6 (AAR27824.1); CHMO-Rphi1, *Rhodococcus* sp. Phi1 (AAN37494.1); CHMO-RPhi2, *Rhodococcus* sp. Phi2 (AAN37491.1); BVMO3 from *Dietzia* sp. D5 (AHE80562.1), MO0565c-Mt from *Mycobacterium tuberculosis* H37Rv (NP_215079.1), ETHA3845c-Mt from *Mycobacterium tuberculosis* H37Rv (NP_218371.1), MO16-Rj from *Rhodococcus jostii* RHA1 (YP_702882.1), BVMO-Pp from *Pseudomonas putida* KT2440 (NP_744949.1), MO13-Mt *Rhodococcus jostii* RHA1 (YP_703731.1), MO3083-Mt from *Mycobacterium tuberculosis* H37Rv (NP_217599.1), BVMO-Ar from *Acinetobacter radioresistens* (ADF32068), BVMO-Pf from *Pseudomonas fluorescens* DSM50106 (AAC36351.2), CHMO-Bp, *Brachymonas petroleovorans* (AAR99068.1), BVMO-Php, *Physcomitrella patens* BVMO (XP_001758613); BVMO-Cm, *Cyanidioschyzon merolae* BVMO (XP_005536938); BVMO-Pv, *Pseudomonas veronii* MEK700 BVMO (ABI15711); AFL 210, AFL 456, AFL 619 and AFL 838, *Aspergillus flavus* BVMOs (XP_002375343; XP_002375466; XP_002383043; XP_002375657).

Table 1: Sequence identity among the BVMOs considered in Figure 1.
The BVMOs in the cells shaded in green belong to the EthA group.

| Seq 1 | Lenght seq 1 | Seq 2 | Lenght seq 2 | Prot identity (%) |
|-------|--------------|--------------|--------------|-------------------|
| BVMO3 | 521 | ETHA3845c-Mt | 489 | 42.13 |
| BVMO3 | 521 | MO16-Rj | 498 | 42.97 |
| BVMO3 | 521 | BVMO-Pp | 508 | 41.93 |
| BVMO3 | 521 | MO13-Rj | 515 | 38.45 |
| BVMO3 | 521 | MO0565c-Mt | 486 | 40.53 |
| BVMO3 | 521 | MO3083-Mt | 495 | 38.99 |
| BVMO3 | 521 | BVMO-Ar | 496 | 37.7 |
| BVMO3 | 521 | PAMO-Tf | 542 | 15.74 |
| BVMO3 | 521 | SMO-Rr | 563 | 17.08 |
| BVMO3 | 521 | CPDMO-PHI-70 | 601 | 14.4 |
| BVMO3 | 521 | BVMO4-D | 612 | 17.27 |
| BVMO3 | 521 | CPMO-C | 550 | 16.7 |
| BVMO3 | 521 | BVMO-Pa | 527 | 18.23 |
| BVMO3 | 521 | BVMO-Pf | 512 | 18.95 |
| BVMO3 | 521 | HAPMO-Pf | 640 | 16.12 |
| BVMO3 | 521 | HAPMO-Pp | 640 | 19.58 |
| BVMO3 | 521 | CHMO-Ac | 543 | 16.7 |
| BVMO3 | 521 | CHMO-RHI-31 | 540 | 20.54 |

Paper III

ORIGINAL ARTICLE

Open Access

Cloning, expression and characterization of a versatile Baeyer-Villiger monooxygenase from *Dietzia* sp. D5

Serena Bisagni, Rajni Hatti-Kaul and Gashaw Mamo*

Abstract

A novel BVMO encoding gene was identified from a draft genome sequence of a newly isolated strain of *Dietzia*. Analysis of the protein sequence revealed that it belongs to a group of BVMOs whose most characterized member is cyclopentadecanone monooxygenase (CPDMO). The gene was PCR amplified, cloned and successfully expressed in *E. coli*. The expressed recombinant enzyme was purified using metal affinity chromatography. Characterization of the purified enzyme revealed that it has a broad substrate scope and oxidized different compounds including substituted and unsubstituted alicyclic, bicyclic-, aliphatic-ketones, ketones with an aromatic moiety, and sulfides. The highest activities were measured for 2- and 3-methylcyclohexanone, phenylacetone, bicyclo-[3.2.0]-hept-2-en-6-one and menthone. The enzyme was optimally active at pH 7.5 and 35°C, a temperature at which its half-life was about 20 hours. The stability studies have shown that this enzyme is more stable than all other reported BVMOs except the phenylacetone monooxygenase from the thermophilic organism *Thermobifida fusca*.

Keywords: Baeyer-Villiger monooxygenase; Biocatalysis; Enzyme stability; Protein expression

Introduction

Baeyer-Villiger reaction was discovered more than one hundred years ago and refers to the oxidation of a ketone to a lactone or an ester (Baeyer and Villiger 1899). It is regarded as one of the important reactions in chemical industry and is currently accomplished in organic solvents using peroxyacids as oxidants. However, these compounds are harmful and explosive; hence it is considered that Baeyer-Villiger reaction using these oxidants is problematic and unsafe in large-scale industrial reactions (Stewart 1998). Discovery of enzymes known as Baeyer-Villiger monooxygenases (BVMOs) which are able to catalyze such reactions has led to possible alternative of using them as safe and green catalysts for Baeyer-Villiger reactions. In line with this, in the last fifteen years, more than fifty BVMOs have been cloned and expressed in heterologous systems and a number of other BVMOs have also been directly purified from different wild type microorganisms and characterized (Leisch et al. 2011).

BVMOs are flavin-dependent enzymes which require NADPH for regeneration of FAD and use molecular oxygen as the oxidizing agent. These enzymes are known to catalyze the oxidation of ketones and heteroatoms such as sulphur, nitrogen, boron, phosphorus and selenium (Walsh and Chen 1988). In addition to safety, the use of BVMOs allows to run reactions with high enantio- and regio-specificity, which is often difficult to achieve when chemical catalysts are employed. In applications where the chiral nature of the product is an important factor such as in pharmaceutical industries (Pollard and Woodley 2007; Ramesh 2008), the use of BVMOs is of great interest. However, despite such advantages, the application of BVMOs in industrial processes (Baldwin et al. 2008) is hampered due to lack of robust enzymes, complexity of the reaction processes, such as substrate and product inhibition, and difficult downstream processing. One of the major bottlenecks is the poor stability of BVMOs (Leisch et al. 2011). With the exception of phenylacetone monooxygenase (PAMO) obtained from a strain of the thermophilic bacterium *Thermobifida fusca* (Fraaije et al. 2005), all the other known BVMOs are not stable and very rapidly lose activity even at room temperature (Völker et al. 2008;

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Rehdorf et al. 2009; Kadow et al. 2012). Moreover, the activity and stability of BVMOs are known to be affected by organic solvents (de Gonzalo et al. 2006; Secundo et al. 2011). More stable BVMOs can be potentially obtained by rigorous protein engineering work, and new enzyme discovery by means of conventional microbial screening or from metagenome libraries and genome sequences.

In this article, we describe the cloning, expression and characterization of a novel BVMO from a strain of *Dietzia*, an organism previously isolated from a soda lake water sample. Although a number of *Dietzia* were isolated and reported to have rich oxygenase diversity, there has been no BVMO isolated and characterized from these organisms.

Materials and methods

Chemicals

All chemicals were purchased from Sigma Aldrich (Stockholm, Sweden), Calbiochem (Darmstadt, Germany) and VWR (Stockholm, Sweden). All the chemicals for PCR, T4 DNA ligase and FastDigest® restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). QIAGEN Plasmid Mini Kit and QIAEX II Gel Extraction Kit were purchased from Qiagen (Sollentuna, Sweden).

Microorganisms and plasmid

Dietzia sp. D5 isolated in our laboratory is deposited in Culture Collection, University of Göteborg (CCUG 64924), Sweden and its genomic DNA was purified using ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, USA). *E. coli* NovaBlue, BL21(DE3), Rosetta2(DE3) and the plasmids pET-22b(+) were purchased from Novagen (Darmstadt, Germany). *E. coli* BL21-CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP cells were purchased from Agilent Technologies (Santa Clara, USA).

Gene analysis and cloning

The gene encoding the monooxygenase BVMO4 was identified from the draft genome sequence of *Dietzia* sp. D5 (unpublished data). Identification of ORFs and analysis of the BVMO4 gene and its deduced protein sequence was performed with CLCBio Main Workbench (Aarhus, Denmark) FGENESB (Soft Berry Mount Nysco, USA) and BLASTp at NCBI. The phylogenetic tree was generated using the software FigTree.

The BVMO4 encoding gene was amplified by PCR from *Dietzia* sp. D5 genomic DNA sample using a pair of primers, forward primer ATTACCATGGCCTTCAC CCTCCCTG and reverse primer ATTAGCGGCCGCG GCCACCGGGACCGCGTCG which have the *Nco*I and *Not*I restriction sites, respectively. The PCR amplification was performed with High Fidelity PCR Enzyme Mix (Fermentas) following the manufacturer protocol but supplemented with 2.5% (v/v) DMSO. The PCR product was

purified using PCR cleaning kit and digested with *Nco*I and *Not*I. The digested DNA was loaded on agarose gel and after electrophoresis it was extracted from the gel using Qiaex II gel extraction kit, and ligated to the expression plasmid pET-22b(+) which was digested using *Nco*I and *Not*I. The ligation mix was transformed into competent *E. coli* NovaBlue cells and transferred to ampicillin containing LB-agar plates. After overnight incubation colonies were screened by PCR and plasmids from the insert positive colonies were extracted, sequenced at GATC Biotech AG, Konstanz, Germany and plasmids with correct sequences were transformed to the expression hosts.

Protein expression and purification

Recombinant *E. coli* BL21(DE3), Rosetta(DE3), Arctic Express(DE3)-RP and BL21-CodonPlus(DE3)-RP cells were grown in LB medium containing, whenever required, ampicillin 100 µg/ml, chloramphenicol 34 µg/ml and gentamycin 20 µg/ml, respectively. The cultures were incubated at 30°C with shaking (180 rpm), until the OD₆₀₀ reached 0.6, then induced with 1 mM IPTG. After overnight induction at 15°C, the cultures were harvested by centrifugation, resuspended in 20 mM sodium phosphate buffer, pH 7.4 and disrupted by sonication for three rounds of 45 sec burst at 50% amplitude and 50% cycle, and 1 min break. The cell homogenate was centrifuged at 4°C for 15 minutes at about 15000 g using a Sorvall centrifuge and the clear supernatant used as source of the recombinant enzyme.

The His-tagged recombinant enzyme was purified at 4°C by Ni-NTA affinity chromatography using HisTrap™ FF crude column (GE Healthcare, Uppsala, Sweden) following the manufacturer instructions. After elution from the column, the enzyme was desalted and concentrated using Vivaspin 20 MWCO 30,000 centrifugal concentrators (Sartorius Stedim Biotech GmbH, Goettingen, Germany), FAD was added to a final concentration of 10 µM and stored at 4°C in 50 mM sodium phosphate buffer, pH 7.5. The homogeneity of purified sample was checked using 10% SDS-PAGE prepared according to Laemmli (Laemmli 1970).

Enzyme assay

BVMO activity was measured by monitoring the decrease in absorbance of NADPH at 340 nm ($\epsilon_{\text{NADPH340}} = 6.22 \text{ cm}^{-1} \text{ mM}^{-1}$) after addition of the substrate. Exceptions were made for 4'-hydroxyacetophenone that strongly absorbs at 340 nm and for which NADPH depletion was measured at 370 nm ($\epsilon_{\text{NADPH370}} = 2.7 \text{ cm}^{-1} \text{ mM}^{-1}$), and ethionamide for which the product formation was measured at 400 nm ($\epsilon_{\text{ETH400}} = 1.0 \text{ cm}^{-1} \text{ mM}^{-1}$) instead of cofactor depletion. All spectrophotometric measurements were made using UV-1650 PC Spectrophotometer (Shimadzu, Kyoto, Japan) at 25°C, unless otherwise

mentioned. The reactions were done in 50 mM sodium phosphate buffer pH 7.5, containing 10 mM KCl, 60 μ M NADPH and 5 mM substrate (except steroids which were used at 0.5 mM due to their low solubility). The final enzyme concentration in the assay was 0.024 mg/ml. Steady state kinetics were measured by varying either the cofactor NADPH or the substrate (phenylacetone or 2-methylcyclohexanone). The kinetic parameters (K_m and k_{cat}) of the BVMO were determined using the Lineweaver-Burk plot of the Michaelis-Menten equation under steady-state conditions.

Effect of pH and temperature on BVMO4 activity and stability

Effects of pH and temperature on the enzyme activity were determined by assaying the enzyme at different pH (pH 5–9) and temperatures (10–45°C). To determine the effect of pH on the enzyme stability, the enzyme was

kept in the buffers at 4°C for 24 days and the residual activity was determined. Similarly, thermal stability of BVMO4 was studied by incubating the enzyme at 35°C in 50 mM sodium phosphate buffer, pH 7.5 and measuring the residual activity of samples withdrawn periodically. The enzyme solutions used to determine the enzyme stability was 0.5 mg/ml.

Effect of freezing-thawing on BVMO4 activity

The enzyme solution (0.5 mg/ml) was subjected to freeze-thaw cycles in the presence of various cryoprotectants, added at a final concentration: glycerol 20% (v/v), sorbitol 1.5 M, trehalose 0.5 M, betaine 1.5 M, DMSO 20% (v/v), 2-methyl-2,4-pentanediol 20% (v/v), PEG 600 20% (w/v), BSA 20% (w/v), ectoine 0.5 M, hydroxyectoine 0.5 M. The enzymatic activity was measured (using phenylacetone as substrate) before freezing and after freeze-thaw cycles.

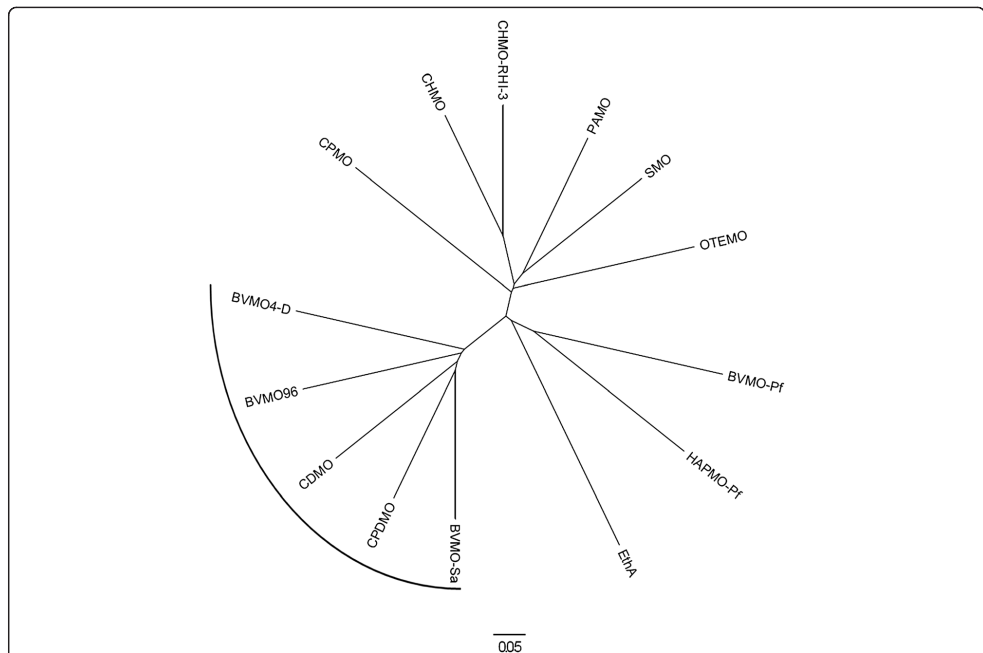
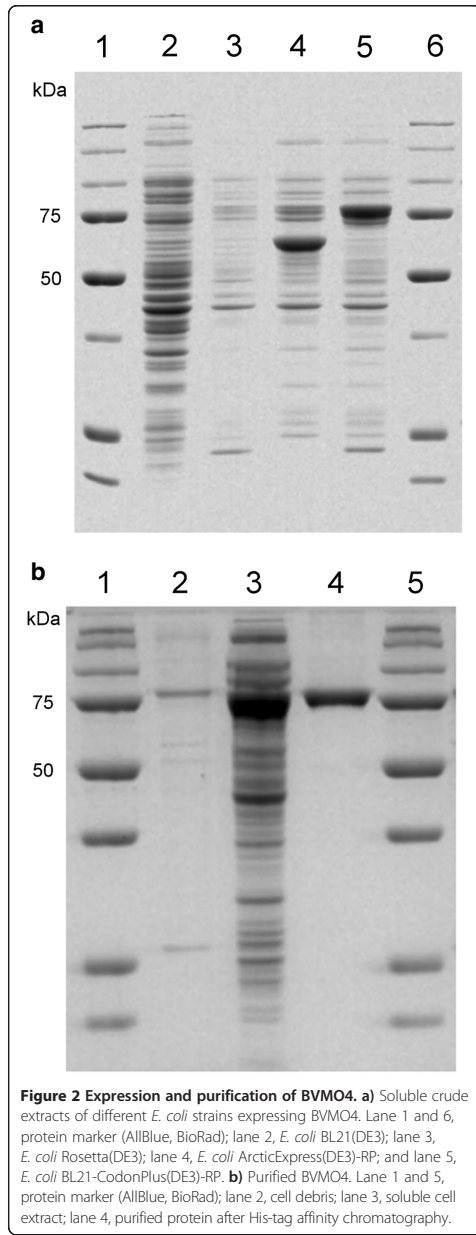


Figure 1 A phylogenetic tree of BVMOs. The alignment was done using T-Coffee and the phylogenetic tree was derived using Clustal W2. The BVMOs represented on the tree are: BVMO4 *Dietzia* sp. D5 (AGY78320.1); BVMO96 *Streptomyces coelicolor* A3(2) (NP_624628.1); CDMO *Rhodococcus ruber* SC1 (AAL14233.1); CPDMO *Pseudomonas* sp. HI-70 (BAE93346.1); BVMO-Sa is BVMO *Streptomyces avermitilis* MA-4680 (NP_824170.1); EthA is EthA3854c *Mycobacterium tuberculosis* H37Rv (NP_218371.1); HAPMO-Pf is HAPMO *Pseudomonas fluorescens* ACB (Q93TJ5.1); BVMO-Pf is BVMO *Pseudomonas fluorescens* DSM50106 (AAC36351.2); OTEMO *Pseudomonas putida* (3UOV_A); SMO *Rhodococcus rhodochrous* (BAF48129.1); PAMO *Thermobifida fusca* YX (YP_289549.1); CHMO-RHI-3 is CHMO *Rhodococcus* sp. HI-31 (3UCL_A); CHMO *Acinetobacter* sp. NCIMB9871 (BAA86293.1); CPMO *Comamonas* sp. NCIMB9872 (Q8GAW0.3).



BVMO4 activity in the presence of salt and organic solvents

To determine salt tolerance, the enzyme activity on phenylacetone was measured at pH 7.5 in the presence of 0-1.2 M NaCl. Similarly, the effect of organic solvents on the activity of the enzyme was determined by measuring the activity in the presence of 20% (v/v) organic solvents.

Nucleic acid sequences

The nucleic acid and protein sequences reported in this work are available at GenBank under the deposition number KF319017.

Results

Gene and protein sequence analysis

The gene encoding BVMO4 was amplified from the genomic DNA of *Dietzia* sp. D5. The enzyme is composed of 612 amino acids, which makes it larger than most BVMOs which are 500 to 550 amino acids long. BLAST search against database sequences revealed highest identity (63%) with the unstudied putative BVMO from *Gordonia terrae* NBRC 100016 (ZP_09801205), while among characterized BVMOs, 41-44% sequence similarity was observed with a group of BVMOs whose most studied member is CPDMO and that like BVMO4 are about 600 amino acids long in their primary structure. A phylogenetic tree of BVMO4, CPDMO, CPDMO-like and other known BVMOs is shown in Figure 1.

Comparison of the DNA sequence of the genome fragment of *Dietzia* sp. D5 containing BVMO4 gene with the recently published genome of a closely related organism, *Dietzia cinnamea* P4 (Procópio et al. 2012) showed that most of the genes neighboring the BVMO gene are present in the two organisms and have the same order, although two of the predicted ORFs in *Dietzia* sp. D5 are missing in *D. cinnamea* P4. The missing ORFs are the ones encoding BVMO4 and a TetR transcriptional regulator gene.

Expression and purification of BVMO4

E. coli strains BL21(DE3), Rosetta(DE3), ArcticExpress (DE3)-RP and BL21-CodonPlus(DE3)-RP were used to evaluate the expression of BVMO4 in soluble form. The best result was achieved with *E. coli* BL21-CodonPlus

Table 1 Summary of the recombinant BVMO4 purification steps

| Steps | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification fold |
|-----------------|--------------------|--------------------|--------------------------|-------------------|
| Crude extract | 78.86 | 2.95 | 0.037 | 1 |
| Purified enzyme | 4.95 | 3.39 | 0.648 | 17.5 |

(DE3)-RP and SDS-PAGE of the crude cell extract shows a prominent band at about 75 kDa (Figure 2a) which is close to the *in silico* predicted mass of 72 kDa. *E. coli* BL21(DE3) cells were unable to express BVMO4 while *E. coli* Rosetta(DE3) and ArcticExpress(DE3)-RP resulted in modest levels of expression; however, the cell growth was considerably lower when compared to BL21-CodonPlus(DE3)-RP. Thus, BL21-CodonPlus(DE3)-RP was chosen as the expression host. In the case of *E. coli* ArcticExpress(DE3)-RP the thick protein band at approximately 55 kDa in Figure 2a is a chaperone that is expressed to help the folding of the BVMO.

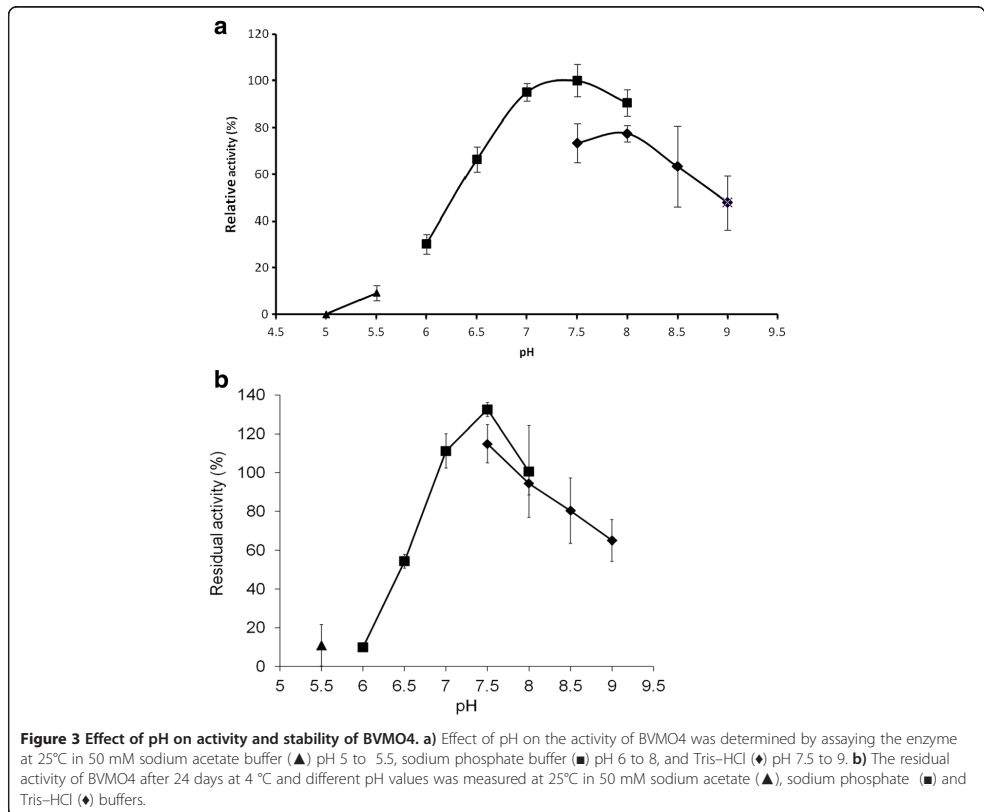
The enzyme was purified by His-tag affinity chromatography and the summary of the enzyme purification steps is given in Table 1. The enzyme lost its activity during the purification process but was recovered upon addition of FAD. The specific activity of the pure enzyme

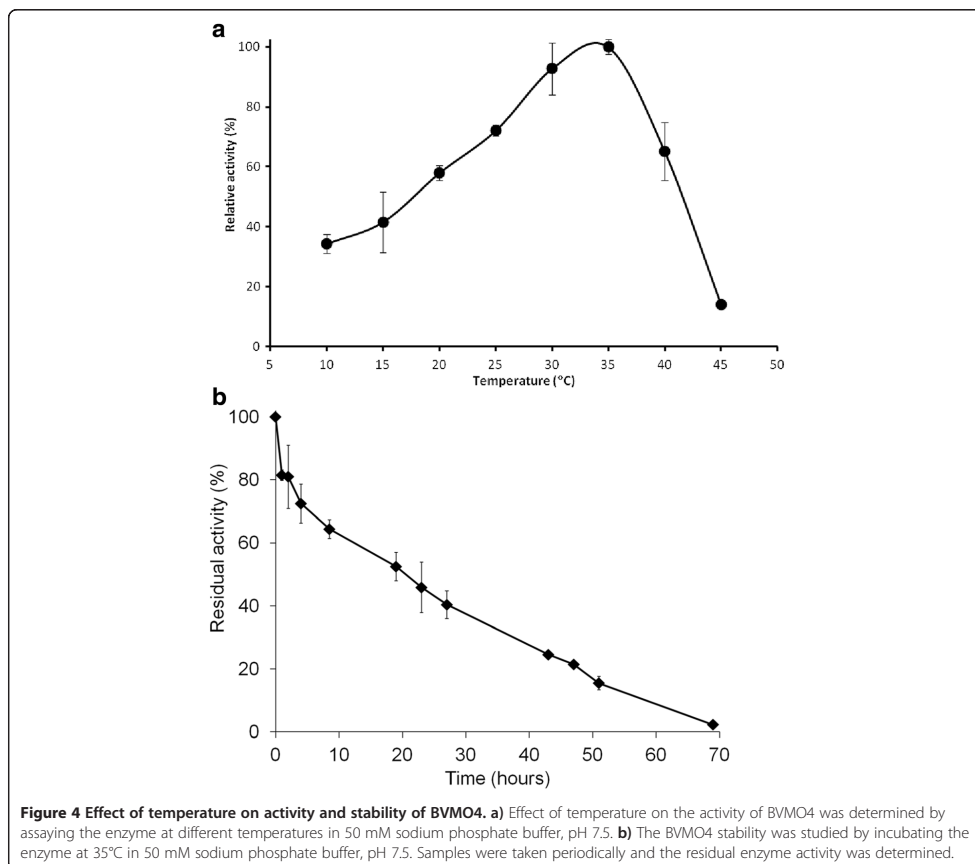
was about 0.65 U/mg protein when assayed using phenylacetone as substrate.

Effect of pH and temperature on activity and stability of BVMO4

Effect of pH on the activity of the pure enzyme was determined by performing the assay in a pH range from 5 to 9. The enzyme was optimally active at pH 7.5 and exhibited more than 50% of its optimal activity between pH 6.5 and 9.0 (Figure 3a). When stored at 4°C, the enzyme displayed highest stability in a pH range of 7.0–8.0 (Figure 3b).

The enzyme was optimally active at 35°C (Figure 4a) and retained about 50% and 20% of its initial activity after 20 h and 48 h of incubation at 35°C, respectively (Figure 4b). The recombinant BVMO can be stored frozen at -20°C for several months without loss of activity.





Moreover frozen aliquots of the enzyme proved to be resistant to at least three freeze-thaw cycles both in the presence and absence of cryoprotectants. However, the enzyme lost most of its activity when 2-methyl-2,4-pentanediol was used as cryoprotectant (data not shown).

Determination of BVMO4 substrate scope

A total of 28 compounds were studied as substrates for BVMO4 (Table 2). Activity on phenylacetone was used as a reference (100%) to determine the relative activity of the enzyme on these different substrates. The highest activity was measured for 2- and 3-methylcyclohexanone, phenylacetone, bicyclo[3.2.0]hept-2-en-6-one and menthone. It was noted that the position of the methyl substitution in the series of methylcyclohexanones influenced the enzyme activity: 2-methylcyclohexanone had the higher relative activity

(155%), 3-methylcyclohexanone was a moderately good substrate (90% relative activity) and 4-methylcyclohexanone reacted poorly (15% relative activity). Among alicyclic ketones, the highest activity was achieved when cyclohexanone was used as substrate, though they do not appear to be among the best substrates for BVMO4. Aliphatic ketones show approximately the same reactivity as cyclohexanone regardless of the chain length. The enzyme oxidized phenylacetone readily but there was little or no detectable activity when the substrate was a related structure, such as acetophenone and 4'-hydroxyacetophenone, which indicates the high selectivity of BVMO4 among these substrates. In addition, there was no detectable activity on benzaldehyde. Its activity on sulfides and ketosteroids, was moderate, and the oxidation on the D ring of ketosteroids seems preferred. A detailed study on the activity and enantioselectivity of BVMO4

Table 2 Relative activity of BVMO4 towards different substrates

| Substrates | Relative activity % |
|--|---------------------|
| Alicyclic ketones | |
| Cyclobutanone | 20.2 ± 6.1 |
| Cyclopentanone | 12.7 ± 5.9 |
| Cyclohexanone | 22.0 ± 3.5 |
| Cycloheptanone | 12.0 ± 3.6 |
| Cyclooctanone | 9.3 ± 3.9 |
| Cyclopentadecanone | 0.0 |
| Substituted alicyclic ketones | |
| 2-methylcyclohexanone | 155.3 ± 24.8 |
| 3-methylcyclohexanone | 90.5 ± 1.9 |
| 4-methylcyclohexanone | 14.6 ± 0.7 |
| 2-cyclohexen-1-one | 5.4 ± 1.1 |
| Bicyclic ketones | |
| Bicyclo[3.2.0]hept-2-en-6-one | 103.0 ± 6.4 |
| Norcamphor | 6.3 ± 2.2 |
| Beta tetralone | 0.0 |
| Alpha tetralone | 10.8 ± 2.7 |
| Aliphatic ketones | |
| 2-heptanone | 20.7 ± 0.7 |
| 2-octanone | 22.6 ± 1.7 |
| 2-decanone | 22.0 ± 0.4 |
| 2-pentadecanone | 20.9 ± 3.5 |
| Aliphatic ketone with aromatic substituents | |
| Phenylacetone | 100.0 ± 6.2 |
| Acetophenone | 2.4 ± 0.8 |
| 4'-hydroxyacetophenone | 0.0 |
| Benzaldehyde | 0.0 |
| Sulfides | |
| Thioanisole | 33.0 ± 13.5 |
| Ethionamide | 32.0 ± 1.8 |
| Other ketones | |
| Progesterone ^a | 23.0 ± 0.6 |
| Estrone ^a | 17.0 ± 0.4 |
| Testosterone ^a | 6.6 ± 2.4 |
| Menthone | 77.1 ± 2.7 |

The activity on phenylacetone which is considered as 100% was 1.87 mU. The initial substrate concentration was 5 mM. ^aSubstrate concentration was 0.5 mM.

with sulfide and aldehyde substrates has been reported elsewhere (Bisagni et al. 2014).

Steady state kinetic properties of BVMO4 were determined for the cofactor NADPH and two of the best substrates, 2-methylcyclohexanone and phenylacetone (Table 3). The enzyme displayed relatively higher affinity (lower K_m) for 2-methylcyclohexanone but higher V_{max} and k_{cat} for phenylacetone as a result of which k_{cat}/K_m were similar for the two substrates. The K_m for NADPH was about 10 μ M while there was no detectable activity when NADH was used as a cofactor, which indicates that BVMO4 is strictly NADPH dependent.

Enzyme activity in presence of sodium chloride and organic solvents

Dietzia sp. D5, the source organism for BVMO4, as well as other *Dietzia* spp., are halotolerant (Plakunov et al. 2008) and hence the salt tolerance of the enzyme was investigated. At 0.4 M NaCl, the enzyme exhibited more than 50% of the activity in the absence of salt, and the activity decreased steadily with increasing salt concentration up to 1.2 M, at which there was no detectable activity (Figure 5a).

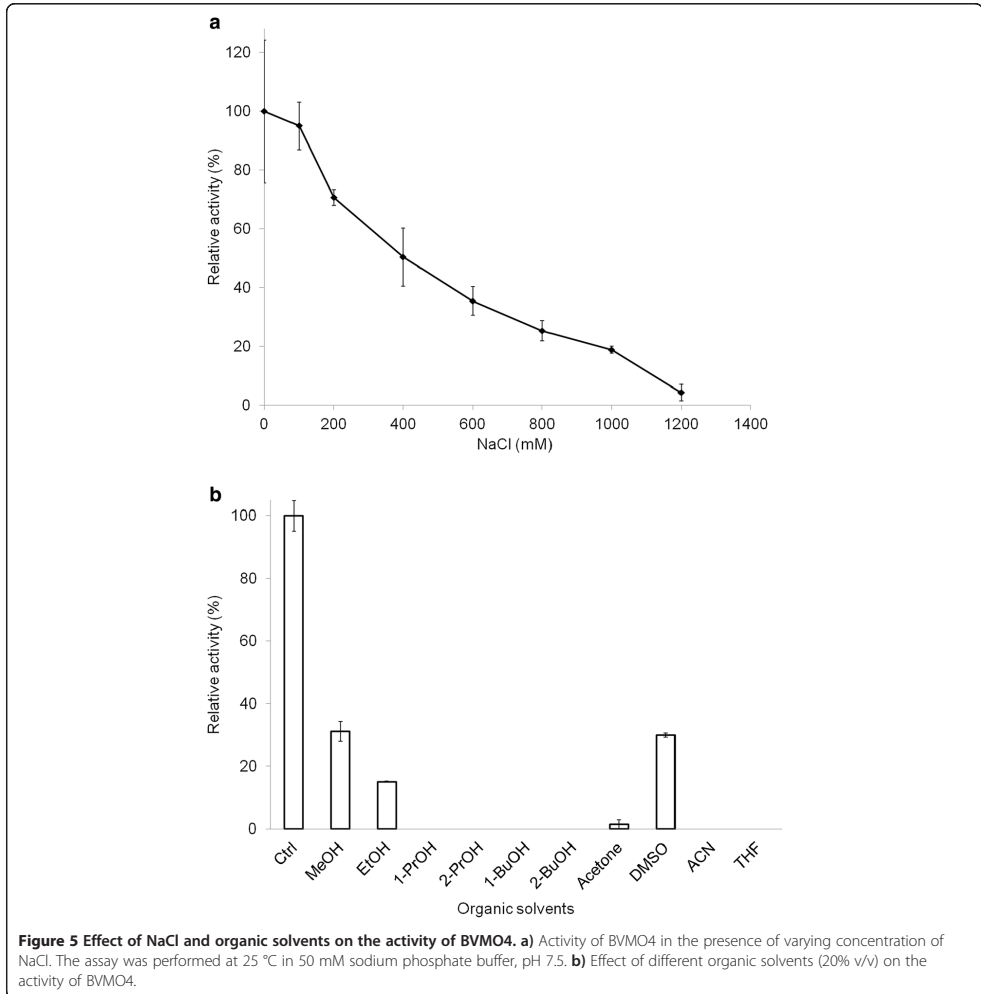
The influence of organic solvents frequently used in organic synthesis, on BVMO4 activity was also investigated. The solvents were added to the reaction mixture at a final concentration of 20% (v/v). Some activity was detected in the presence of methanol, DMSO and to a lesser extent ethanol but there was no detectable activity in the other solvents tested (Figure 5b).

Discussion

The sequence similarity analysis of BVMO4 revealed that it is related to a group of Baeyer-Villiger monooxygenases which are characterized by longer amino acid sequences compared to most other BVMOs. The most studied member of this group of BVMOs is CPDMO, a BVMO from *Pseudomonas* sp. HI-70 (Iwaki et al. 2006; Beneventi et al. 2009; Fink et al. 2011). The other BVMOs belonging to this group are both of microbial and fungal origin (Miyamoto et al. 1995; Jiang et al. 2009; Qiao et al. 2011) and are known to be very versatile in terms of their substrate scope, and efficiently catalyze the oxidation of a wide range of substrates, which indicates a broader application potential (Fink et al. 2012; Bianchi et al. 2013).

Table 3 Steady state kinetic properties of BVMO4

| | K_m (mM) | V_{max} (mM s ⁻¹) | k_{cat} (s ⁻¹) | k_{cat}/K_m (mM ⁻¹ s ⁻¹) |
|-----------------------|---------------|---------------------------------|------------------------------|---|
| Phenylacetone | 0.829 ± 0.163 | 7.90E-05 ± 2.31E-06 | 0.634 ± 0.104 | 0.77 ± 0.03 |
| 2-methylcyclohexanone | 0.507 ± 0.142 | 5.11E-05 ± 1.31E-05 | 0.370 ± 0.095 | 0.73 ± 0.02 |
| NADPH | 0.011 ± 0.002 | 7.00E-05 ± 7.80E-06 | 0.507 ± 0.057 | 45.06 ± 1.52 |



Dietzia spp. genomes have high GC content; in fact the BVMO4 encoding gene has over 67% GC content and a number of rare codons. Expression of high GC genes containing multiple rare codons in *E. coli* is difficult and most of the commonly used strains such as *E. coli* BL21(DE3) cannot properly express such genes. However, some *E. coli* strains developed for the expression of this kind of genes are appearing in the market. Among these strains are *E. coli* Rosetta(DE3), BL21-CodonPlus(DE3)-RP and ArcticExpress(DE3)-RP which successfully expressed BVMO4 gene as shown in Figure 2a while expression in BL21(DE3) was not possible. BL21-

CodonPlus(DE3)-RP was better than the other two strains; protein expression is much higher and the culture has grown to cell density comparable to that of BL21(DE3), while the other strains, especially Rosetta(DE3), have a much lower growth rate.

The recombinant BVMO4 expressed in *E. coli* BL21-CodonPlus(DE3)-RP cells was purified to homogeneity by immobilized metal ion affinity chromatography. It was observed that the enzyme completely lost its activity during the purification process. Since the activity of the purified enzyme was recovered with addition of FAD, the loss of activity during purification is believed to be

due to loss of the cofactor and such phenomenon has been reported before (Nam et al. 2002; Malito et al. 2004). Addition of FAD after the enzyme purification not only restored the activity but also resulted in a higher level of total activity in the purified enzyme than the crude form (Table 1). This might be due to an insufficient production of FAD by the *E. coli* cells, which may result in a part of the expressed enzyme in the crude extract being inactive due to lack of the cofactor. The addition of FAD precursors such as riboflavin, to the reaction media might increase the enzymatic activity in the crude extract (Yoshikane et al. 2004; Wang and Wang 2007).

The optimal pH for the activity of BVMO4 (pH 7.5) is slightly lower than the pH optima of most BVMOs that lies between pH 8 and 9.5. However, it is similar to that of cyclopentanone monooxygenase (pH 7.7) from a *Pseudomonas* strain (Griffin and Trudgill 1976). BVMO4 is less active and less stable in acidic solution which has also been observed for other BVMOs (Secundo et al. 2005; Völker et al. 2008; Rehdorf et al. 2009).

Although several BVMOs are available as recombinant enzymes, they have so far been very seldom used in industrial applications (Baldwin et al. 2008). Poor stability is one of the factors that hindered their applications (Clouthier and Pelletier 2012). A number of purified monooxygenases rapidly lose activity even when stored at 4°C or in a frozen state (Völker et al. 2008; Kadow et al. 2012). A CHMO from *Acinetobacter*, one of the most studied BVMOs, has a half-life of 24 hours at 25°C (Zambianchi et al. 2002) and a similar property was observed for a HAPMO (Rehdorf et al. 2009). Another enzyme, OTEMO is totally inactive after 4 hours at 25°C and loses half of its initial activity within 24 hours at 4°C (Kadow et al. 2012). Interestingly, BVMO4 displayed higher stability than the great majority of BVMOs. Moreover, BVMO4 can be stored and freeze-thawed repeatedly without significant loss of activity.

As applications of BVMOs involve oxidation of organic compounds, it is important to know the effect of organic solvents, such as those used to dissolve substrates, on the enzyme activity. The activity of BVMO4 in the presence of organic solvents at 20% (v/v) final concentration was comparable to what has been reported for a CHMO (Secundo et al. 2011) but lower than a PAMO (de Gonzalo et al. 2006, 2012). Similarly, high salt concentration can affect enzyme hydration and enzymes that are stable at high salt concentrations are preferred for industrial applications (Woodley 2013). BVMO4 retained more than 50% of its salt-free activity at NaCl concentration of up to 0.4 M. Although this concentration does not seem outstandingly high, the enzyme has shown moderate resistance. Such data is however not available for other BVMOs.

BVMO4 has a wide substrate scope and oxidizes substrates with aromatic moiety, substituted cyclic ketones and ketones in multi-ring compounds. In addition, the enzyme oxidized alicyclic and linear aliphatic ketones, thiols and bulky substrates, such as steroids, although at a lower rate. Despite the primary sequence of the enzyme is close to BVMOs belonging to CPDMO group, it poorly oxidizes medium-sized ketones and did not show activity on cyclopentadecanone. Some discrepancies in the enzyme sequence can be the cause for the different affinity for bulky alicyclic ketones. Thus, it can be speculated that BVMO4 is a novel enzyme. In fact, when compared to other BVMOs in the CPDMO group, this enzyme branches out early in the phylogenetic tree (Figure 1). When the kinetic parameters were measured with phenylacetone and 2-methylcyclohexanone as substrates, the K_m values were in the order of hundreds of μM (Table 3). The K_m of cyclohexanone for CHMO (Trudgill 1990) is two orders of magnitude lower than the ones measured for BVMO4, indicating that although phenylacetone and 2-methylcyclohexanone are readily oxidized, BVMO4 has relatively low affinity for the substrates.

The results of this study show that BVMO4 is an interesting enzyme to catalyze oxidation of various substrates. In particular, its wide substrate scope and high stability make this enzyme a potential candidate for various Baeyer-Villiger oxidations. Further work to demonstrate its potential on biotransformations of different substrates, understanding its structure-function relationship and improving its property through mutagenesis is currently being considered.

Competing interests

The authors declare that they have no competing interests.

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Paper IV

Exploring the Substrate Specificity and Enantioselectivity of a Baeyer–Villiger Monooxygenase from *Dietzia* sp. D5: Oxidation of Sulfides and Aldehydes

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Abstract Baeyer–Villiger monooxygenases (BVMOs) are valuable enzymes for specific oxyfunctionalization chemistry. They catalyze the oxidation of ketones to esters, but are also capable of oxidizing other chemical functions, namely aldehydes and heteroatoms such as sulfur, nitrogen, selenium and boron. The oxidation specificity and enantioselectivity of a newly characterized BVMO (BVMO4) from a strain of *Dietzia* towards sulfide- and aldehyde substrates have been studied. BVMO4 could react with sulfides containing an aromatic group. The presence of a substituent on the aromatic group was tolerated when they were in the *meta*- and *para* position and the oxidations yielded predominantly the (*R*)-sulfoxides. Similarly, BVMO4 displayed a higher activity for aldehydes containing a phenyl group, but long aliphatic aldehydes, namely octanal and decanal, were also accepted as substrate by this enzyme. The major oxidation products of the aldehyde substrates were the respective carboxylic acids in contrast to formate ester that was obtained in most of the previous reports. The Baeyer–Villiger oxidation of the substrate 2-phenylpropionaldehyde was studied in further detail and the corresponding acid product was obtained with good regio- and enantioselectivity. This is a unique

feature for BVMO4 and is of great interest for further exploration of an alternative biocatalytic process.

Keywords Baeyer–Villiger monooxygenase · 2-Phenylpropionaldehyde · *Dietzia* · Profen · Biocatalysis

1 Introduction

Baeyer–Villiger monooxygenases (BVMOs) are flavin enzymes that are able to oxidize ketones to the corresponding esters, by reductive activation of molecular oxygen [1, 2]. BVMOs are preferred alternatives over their chemical counterparts because they react under mild conditions and do not require unsafe organic oxidants. Furthermore, the high selectivity enables simple access to enantiopure products. At the moment, BVMOs are seldom used in preparative applications because they are not an economically viable option when it comes to low-value added, bulk- and commodity products but they are promising enzymes to be developed for the synthesis of highly value-added pharmaceutical intermediates [3–6]. In fact, BVMOs are the object of ongoing research in order to tackle the challenges for their application in industrial biocatalytic processes. Particular efforts have been made to provide efficient systems for the regeneration of the cofactor NADPH [7–9], and development of robust, thermostable BVMOs by genome mining and protein engineering [10, 11]. In addition to the regio- and stereoselectivity, another valuable feature of BVMOs is their substrate promiscuity, allowing the oxidation of a wide range of substrates. BVMOs have been known to transform ketone groups into esters [12], but they can also oxidize aldehydes [13]. Other interesting reactions carried out by

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BVMOs are the oxidation of heteroatoms, such as sulfur, nitrogen, selenium and boron [13–15]. The oxidation of sulfides to sulfoxides has been thoroughly investigated [16–18] and is of special interest for a number of drugs, for example esomeprazole, dexlansoprazole and armodafinil, each of which contains a chiral sulfoxide. The oxidation of aldehydes by BVMOs has also been reported [13, 19, 20]. These findings revealed that the oxidation product in most of these reactions is the formate ester, which hydrolyzes further to formate and the respective alcohol. So far, only cyclohexanone monooxygenase (CHMO) is known to oxidize phenylacetaldehyde producing both regioisomers, the formate ester and the carboxylic acid in a ratio of 7:13 [13]. In addition, CHMO is able to transform aliphatic aldehydes into their respective carboxylic acids with absolute regioselectivity. Although this reaction remained practically unexplored, it has great potential because 2-phenylpropionic acid, the oxidation product of 2-phenylpropionaldehyde, is a precursor of ibuprofen and derivatives, such as flurbiprofen, ketoprofen and naproxen. Ibuprofen is a chiral molecule and only the (*S*)-enantiomer has bioactive properties. To date, several enzymatic methods have been explored to obtain enantiopure ibuprofen [21], including enzymatic resolutions by lipases [22, 23] and oxidation with alcohol dehydrogenases [24, 25], however there is no report on the use of oxygenases.

Recently, a new BVMO from *Dietzia* sp. D5, referred to as BVMO4, has been characterized and revealed to have remarkable stability and catalyze various Baeyer–Villiger oxidation reactions. In this report, the activity of BVMO4 with various sulfide- and aldehyde substrates is described.

2 Materials and Methods

2.1 Chemicals

All the chemicals were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands), Acros Chemicals (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany) and Tokyo Chemical Industry (Oxford, United Kingdom).

2.2 Production of BVMO4

The recombinant BVMO4 was expressed in *E. coli* BL21-CodonPlus(DE3)-RP. Briefly, the bacteria were grown at 30 °C for 3 h, then protein expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and cell cultivation continued for 16 h at 15 °C. *E. coli* cells were harvested, resuspended in sodium phosphate buffer 50 mM pH 7.5 and lysed by sonication. The lysed cells were further centrifuged and the soluble part constituted the crude extract used as a source of BVMO4 for the

present study. Protein concentration was measured by Bradford protein assay (Pierce Protein research Products, Etten-Leur, The Netherlands) according to the manufacturer instructions.

2.3 Screening of Substrates

Two different batches of crude extract containing BVMO4 were used for the screening of the enzyme activity towards sulfides and aldehydes. For the screening of sulfide substrates and bicyclo[3.2.0]hept-2-en-6-one, reaction mixtures contained 0.67 U of BVMO4 crude extract preparation in 50 mM sodium phosphate buffer pH 7.5, 1.25 mM nicotinamide NADPH, 4 mM glucose-6-phosphate and 14 U of glucose-6-phosphate dehydrogenase for cofactor regeneration in a total reaction volume of 500 μ l. The substrate was prepared as a 100 mM stock in ethanol and it was added to the reaction mixture at a final concentration of 5 mM. The reactions for the screening of aldehyde substrates contained 0.37 U of BVMO4 crude extract preparation in 50 mM sodium phosphate buffer pH 7.5, 2 mM NADPH and 2 mM substrate, prepared as a 100 mM stock in acetonitrile, in a total reaction volume of 300 μ l. The reactions were performed in 2 ml Eppendorf tubes and incubated with shaking at 25 °C and shake rate of 500 rpm. Control reactions were prepared using crude extract from *E. coli* BL21(DE3) grown under identical conditions as the one expressing BVMO4. Samples were taken at different times (3 h for octanal and decanal; 4 h for phenylacetone, phenylacetaldehyde and 3-propionaldehyde; 7 h for 2-phenylpropionaldehyde; 16 h for all the other aldehydes; 24 h for the sulfides and bicyclo[3.2.0]hept-2-en-6-one) and analyzed. A unit of BVMO4 is defined as the amount of the enzyme that oxidizes one μ mol of bicyclo[3.2.0]hept-2-en-6-one or one μ mol of phenylacetone in sodium phosphate buffer 50 mM, pH 7.5 at 25 °C and 500 rpm shaking.

2.4 Influence of Substrate and Enzyme Concentration on the Conversion of 2-Phenylacetaldehyde

Modified reactions conditions were investigated for the conversion of 2-phenylacetaldehyde. To determine the effect of the enzyme concentration, the amount of BVMO4 crude extract preparation was increased four-fold while the other component of the reaction remained constant. In a similar way, the substrate concentration was increased four-fold to a final concentration of 8 mM to study its effect on conversion. The substrate was added from a stock solution of 1 M 2-phenylpropionaldehyde prepared in acetonitrile, so that the final concentration of the co-solvent in the reaction mixture would be kept <1 % (v/v).

2.5 Analytical Methods

The initial reaction rate of the sulfides and bicyclo[3.2.0]hept-2-en-6-one was measured by gas chromatography using an Agilent HP-6890 machine equipped with HP-5 column (30 m × 0.32 mm × 0.25 μm). Aliquots (250 μl) were mixed with 500 μl ethyl acetate in Eppendorf tubes, vigorously vortexed and centrifuged (13,000 rpm, 3 min) to allow phase separation. The organic phase was separated, dried over MgSO₄ and transferred to a glass vial. For GC analysis the column temperature was kept constant for 10 min at the following temperatures: 70 °C for *tert*-butyl methyl sulfide; 130 °C for bicyclohept[3.2.0]hept-2-en-6-one; 150 °C for benzyl methyl sulfide, methyl *p*-tolyl sulfide, 4-fluoro thioanisole and thioanisole; and 180 °C for the remaining sulfide containing molecules.

For the determination of enantiomeric excess of sulfoxide products, a BGB-173 column (30 m × 0.25 mm × 0.25 μm) was used. The temperature of the column was set as follows: a gradient from 90 to 134 °C with an increase of 1 °C per minute for the oxidation product of bicyclo[3.2.0]hept-2-en-6-one; 140 °C for 155 min for benzyl methyl sulfoxide; 180 °C for 30 min for all the other sulfoxides. To determine the stereochemistry of the sulfoxide products, the results were compared with those previously obtained by Jensen et al. [26].

The initial reaction rate of the aldehydes (with the exception of octanal and decanal) was measured by HPLC on a Shimadzu LC-20 system equipped with a Shimadzu mSPD-20A Photo Diode Array detector using Xterra column (Rp18 3.5 μM, 4.6 × 150 mm). Aliquots (25 μl) were mixed with 25 μl sodium phosphate buffer, 50 μl 10 % (v/v) TFA and 100 μl acetonitrile. This mixture was vortexed, centrifuged for 3 min at 13,000 rpm and the supernatant was transferred to a glass vial. The compounds were separated using an isocratic flow of two solutions, Solution A (5 % (v/v) acetonitrile, 95 % (v/v) MilliQ water, 0.1 % (v/v) trifluoroacetic acid (TFA)) and Solution B (95 % (v/v) acetonitrile, 5 % (v/v) MilliQ water, 0.1 % (v/v) TFA). The ratio of A:B used for the different substrates was: 100:0 for phenylacetaldehyde; 85:15 for 2-phenylpropionaldehyde; 75:25 for benzylacetaldehyde, 3-phenylpropanal, cinnamaldehyde, cyclohexencarboxaldehyde, indole-3-carboxaldehyde; 50:50 for perillaldehyde and myrtenal.

For determination of the enantiomeric excess of 2-phenylpropionic acid, aliquots (100 μl) were mixed with 50 μl 10 % (v/v) TFA and 200 μl *n*-heptane. The mixture was vigorously vortexed for 45 s and centrifuged for 3 min at 13,000 rpm. The organic phase was dried over MgSO₄ and transferred to a plastic vial and then chromatographed on a HPLC equipped with Chiralpak AD-H column (4.6 × 250 mm, 5 μm) at 40 °C with an isocratic flow of

1 ml/min of 97 % (v/v) *n*-heptane, 3 % (v/v) isopropanol and 0.1 % (v/v) TFA.

For the determination of the enantiomeric excess of 1-phenylethanol, aliquots of the reaction (50 μl) were mixed with 50 μl of 10 % (v/v) TFA and 200 μl of ethyl acetate. Samples were vigorously vortexed for 45 s and centrifuged for 3 min at 13,000 rpm. The organic phase was separated, dried over MgSO₄ and transferred to a glass vial. Gas chromatography of the sample was performed using Shimadzu GC-2010 Plus equipped with CP Chirasil DexCB (25 m × 0.32 mm × 0.25 μm) using the following program: a gradient from 50 to 130 °C with an increase of 40 °C/min, hold at 130 °C for 13 min and from 130 °C to 245 °C with an increase of 60 °C/min.

The initial reaction rate of octanal, decanal and phenylacetone was measured by gas chromatography using Shimadzu GC 2014 with a CP SIL 5CB column (50 m × 0.53 mm × 1 μm) with the following program: hold at 120 °C for 4 min and a gradient from 120 to 335 °C with an increase of 45 °C/min. Aliquots of the reaction (25 μl) were mixed with 25 μl sodium phosphate buffer, 50 μl 10 % (v/v) TFA and 200 μl ethylacetate. After 45 s of vigorous vortexing, mixtures were centrifuged for 3 min at 13,000 rpm, the organic phase was separated, dried over MgSO₄ and transferred to a glass vial.

3 Results and Discussion

3.1 Oxidation of Sulfides and Bicyclo[3.2.0]hept-2-en-6-one

In the present study, the crude recombinant enzyme was used to explore its potential for oxidation of selected sulfide and aldehyde substrates. SDS-PAGE analysis showed the overexpressed BVMO4 to be the most abundant protein in the crude extract (data not shown).

In preliminary work, BVMO4 has shown good activity with bicyclo[3.2.0]hept-2-en-6-one, therefore this substrate was used as a positive control for monitoring the enzyme activity as well as to investigate the stereoselectivity of the oxidation products. We observed that bicyclo[3.2.0]hept-2-en-6-one was oxidized to form three products, including both “normal” lactones, where the (1*S*, 5*R*) product has 56 % *ee* over the other enantiomer, and also the (1*R*, 5*S*) “abnormal” lactone is present with high enantiomeric purity (Table 1). Analogous to the enantiomeric excess we can use the concept of regioisomeric excess (*RE*) to describe the predominance of one regioisomer over the other, and in this case *RE* was 53 % in favour of the “abnormal” lactone. Bicyclo[3.2.0]hept-2-en-6-one is known to be readily oxidized by several BVMOs and different ratios between the four possible products have been

Table 1 Screening of sulfide substrates

| Substrate | Initial rate (Units) | <i>ee</i> |
|-----------------------------------|----------------------|--|
| Bicyclo[3.2.0]hept-2-en-6-one | 0.67 | >99 % (1 <i>R</i> ,5 <i>S</i>) "abnormal" 56 % (1 <i>S</i> , 5 <i>R</i>) "normal" |
| Thioanisole | 1.52 | 50 % (<i>R</i>) |
| Methyl <i>p</i> -tolyl sulfide | 0.62 | 83 % (<i>R</i>) |
| Benzyl methyl sulfide | 2.14 | 58 % (<i>R</i>) |
| Ethyl phenyl sulfide | 2.72 | >99 % (<i>R</i>) |
| 2-Chloro thioanisole | NA | – |
| 3-Chloro thioanisole | 0.44 | 17 % (<i>R</i>) |
| 4-Fluoro thioanisole | 4.06 | 88 % |
| 2-Methoxy thioanisole | NA | – |
| 4-Methoxy thioanisole | NA | – |
| 4-Nitro thioanisole | NA | – |
| 2-Methyl thiopyridine | NA | – |
| <i>tert</i> -Butyl methyl sulfide | NA | – |

One unit (U) corresponds to the amount of enzyme that can oxidize one μmol of substrate per minute under standard analysis conditions. Enantiomeric excess (*ee*) was calculated as $(\text{enantiomer1} - \text{enantiomer2})/(\text{enantiomer1} + \text{enantiomer2})$

NA no detectable activity

observed [1]. Although BVMO4 shows closer sequence similarity to cyclopentadecanone monooxygenase (CPDMO) and cyclododecanone monooxygenase (CDMO) they do not yield similar ratio of the four oxidation products; both CPDMO and CDMO have poor regioselectivity (1:1 ratio between “normal” and “abnormal” lactone) [27, 28]. Among the several examples found in literature, the product composition of BVMO4 shows highest resemblance with that of enzymes MO15 and MO16 from *Rhodococcus jostii* RHA1 [29]. These enzymes convert bicyclo[3.2.0]hept-2-en-6-one with enantiopreference similar to BVMO4, while *RE* is 15 % for MO15 in favour of the “normal lactone” and MO16’s *RE* is 44 % and the “abnormal” lactone prevails. Neither MO15 nor MO16 exhibit considerable amino acid sequence similarity with BVMO4; rather they resemble phenylacetone monooxygenase (PAMO) and EthA monooxygenase, respectively.

BVMO4 was tested for the oxidation of several molecules containing a sulfur heteroatom (Table 1). As commonly observed with BVMOs, even BVMO4 was found to readily oxyfunctionalise sulfides. It shows preference for non-substituted aromatic sulfides but tolerates small substituents in *para* (methyl group, fluoro) or *meta* position (chloro). It is notable that the presence of one extra methyl group in the substrate ethyl phenyl sulfide increased significantly the stereospecificity of the oxidation (>99 %) in comparison to thioanisole (50 %) while the reaction rate was comparable.

In recent years, extensive studies have been done on the oxidation of sulfides by BVMOs [17, 18, 30–32] and flavin monooxygenases (FMOs) [26]. As shown in Table 2, some BVMOs, for instance cyclopentanone monooxygenase (CPMO), 3,6-diketocamphane monooxygenase (3,6-DKCMO) and 4-hydroxyacetophenone monooxygenase (HAPMO), produce selectively the (*S*) sulfoxide; especially the latter enzyme showed an exquisite enantioselectivity [18]. Other BVMOs can be either (*R*) or (*S*) selective depending on the substrate used, as in the case of PAMO, 2,5-diketocamphane monooxygenase (2,5-DKCMO) and the flavin monooxygenase from *Stenotrophomonas maltophilia* (smFMO). BVMO4 was predominantly (*R*) selective, similarly to CHMO, although for the latter enzyme there are two reports presenting contradictory results regarding the enantioselectivity of the oxidation of methyl-*p*-tolyl sulfide [30, 33]. Moreover, BVMO4 showed very good *ee* for the resolution of methyl *p*-tolyl sulfide and ethyl phenyl sulphide (Table 2).

3.2 Aldehyde Oxidation

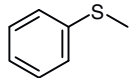
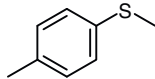
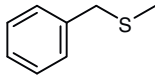
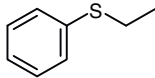
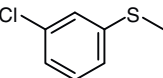
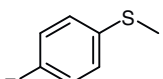
BVMO4 was also used to oxidize substrates containing an aldehyde group from an in-house substrate library. The

results are summarized in Table 3. The monooxygenase was able to oxidize several aromatic and aliphatic compounds containing an aldehyde group. The most remarkable feature of this BVMO is that the major oxidation product is the corresponding carboxylic acid. To date, only CHMO was reported to have such regioselectivity although it was investigated with a limited number of substrates [13]. On the contrary, PAMO produces exclusively the formate ester and even HAPMO yields preferably the ester product [19, 20, 34]. In the case of BVMO4, the regioselectivity varied among the substrates, e.g. 3-phenylpropionaldehyde, octanal and decanal were transformed to the respective acids with exclusive selectivity, while the enzyme showed diminished selectivity with other substrates, such as phenylacetaldehyde, or no regioselectivity, as in the case of 2-phenylpropionaldehyde, where both the formate ester and the acid were formed at similar rates. Noticeably, the only presence of the methyl group in 2-phenylpropionaldehyde reduced the regioselectivity and also the reaction rate by more than three-fold in comparison to phenylacetaldehyde. For all the substrates screened the control reactions showed no oxidation products, with the exception of octanal and decanal where small amounts of oxidation products were measured.

In addition, the mass balance for the oxidation reaction of linear aliphatic aldehydes was not closed, meaning that the sum of the concentrations of substrate and products at the beginning and at the end of the reaction are not constant. In fact about 50 % of the substrates octanal and decanal disappear, probably due to adsorption to the plastic surface of the reaction vessel. The adsorption occurs to a similar extent in the reaction as well as in the control and seems to involve only the substrate and not the products. This adsorption phenomenon was observed also for the other substrates considered in our experiments but to a much less extent, and such behaviour was also reported previously [24]. Alternatively, simple evaporation of the volatile aldehyde substrates may also significantly contribute to the poor mass balances observed so far.

Among the substrates tested, BVMO4 did not oxidize aldehyde groups that are part of a conjugated system (namely, benzaldehyde, cinnamaldehyde, cyclohexencarboxaldehyde, myrtenal, perillaldehyde and indole-3-carboxaldehyde). As observed in case of cinnamaldehyde, the presence of double bond in the side chain makes the oxidation unfavourable, while 3-phenylpropionaldehyde, an identical substrate but without the unsaturation was oxidized most readily. This result suggests that the presence of a conjugated system formed by the aldehyde and a double bond in the α -position to the carbonyl might influence the oxidation of the aldehyde group by the BVMO. Nevertheless testing the oxidation of the saturated form of the other unsaturated substrates present in our screening would be necessary to draw more definitive conclusions.

Table 2 Comparison of the enantioselectivity of BVMO4 with previously reported BVMOs and smFMO

| Substrate | BVMO4 | smFMO | CHMO Ac | CPMO | PAMO | HAPMO | 2,5-DKCMO | 3,6-DKCMO |
|---|----------|----------|------------------------|-----------|----------|----------|-----------|-----------|
|  | 50 % (R) | 21 % (R) | 99 % (R) | 100 % (S) | 44 % (R) | 99 % (S) | 56 % (S) | 11 % (S) |
|  | 83 % (R) | 25 % (R) | 37 % (S) 87 % (R) [33] | 84 % (S) | 10 % (R) | 99 % (S) | 75 % (S) | 40 % (S) |
|  | 58 % (R) | 24 % (R) | 54 % (R) | NR | 94 % (S) | 85 % (S) | NR | NR |
|  | 99 % (R) | 71 % (R) | 47 % (R) | NR | 55 % (S) | 99 % (S) | 8 % (R) | Racemic |
|  | 17 % (R) | 15 % (S) | NR | NR | NR | 96 % (S) | 57 % (S) | 29 % (S) |
|  | 88 % | NR | 92 % (R) | 92 % (S) | NR | NR | NR | NR |

smFMO is flavin monooxygenase from *Stenotrophomonas maltophilia* [26], CHMOAc is cyclohexanone monooxygenase from *Acinetobacter* sp. NCIMB9871[30], CPMO is cyclopentanone monooxygenase from *Comamonas* sp. NCIMB9872 [32], PAMO is phenylacetone monooxygenase from *Thermobifida fusca* YX [17], HAPMO is 4-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* ACB [18], 2,5-DKCMO is 2,5-diketocamphane monooxygenase from *Pseudomonas putida* NCIMB10007 [31] and 3,6-DKCMO is 3,6-diketocamphane monooxygenase from *Pseudomonas putida* NCIMB10007 [31]

NR not reported

Phenylacetone was one of the best substrates for BVMO4 and was hence used as a reference substrate to compare the reactivity of BVMO4 with aldehydes. 3-phenylpropionaldehyde also gave a six-fold higher initial reaction rate as compared to that with phenylacetone. It seems therefore that BVMO4 has very high affinity for phenylaldehydes, especially 3-phenylpropionaldehyde.

Comparison of the results on aldehyde oxidation with BVMO4 in Table 3 with those reported earlier for CHMO highlight a rather similar behaviour of the two enzymes regarding their regioselectivity, that is completely in favour of the acid for linear aliphatic aldehyde substrates and partly in favour of the acid in case of phenylacetaldehyde.

3.3 Influence of the Substrate and Biocatalyst

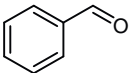
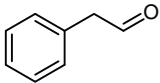
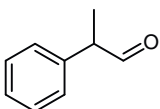
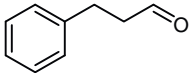
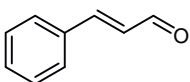
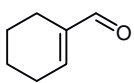
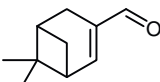
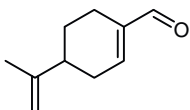
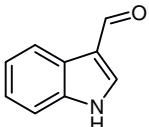
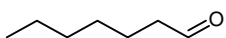
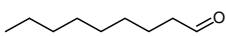
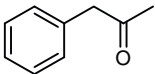
Concentration on the Conversion of 2-Phenylpropionaldehyde

The oxidation of 2-phenylpropionaldehyde by BVMO4 was further investigated. This substrate is especially

interesting because it has a chiral centre that spontaneously undergoes racemisation in slightly alkaline solutions; therefore it is theoretically possible to obtain complete conversion and full enantioselectivity [24]. The reaction scheme is shown in Fig. 1. As observed in Table 3, oxidation of 2-phenylpropionaldehyde produced equal amounts of 2-phenylpropionic acid and 1-phenylethanol, the hydrolysis product or the formate ester.

From our experiments it emerged that substrate and enzyme concentrations can influence the regio- and stereoselectivity of the reaction (Table 4). In the reaction conditions used for the screening there was no preference for the ester or the acid product that were produced in approximately 1:1 ratio, but when the substrate concentration was increased four-fold, the regioselectivity of the enzyme was shifted towards the ester. On the contrary, if the enzyme concentration was increased four-fold the predominant product was the acid. A similar effect was also observed for the enantioselectivity of 1-phenylethanol, the hydrolysis product of the ester, the *ee* of which varied

Table 3 Screening of aldehyde substrates for reactivity with BVMO4

| Substrate | Initial rate (Units) | <i>RE</i> (regioisomeric excess) | Previously reported <i>RE</i> |
|---------------------------|---|----------------------------------|-------------------------------|
| Benzaldehyde |  | NA | – |
| Phenylacetaldehyde |  | 0.84 | 41 % (acid) |
| 2-Phenylpropionaldehyde |  | 0.23 | 2 % (ester) |
| 3-Phenylpropionaldehyde |  | 2.35 | 100 % (acid) |
| Cinnamaldehyde |  | NA | – |
| Cyclohexencarboxaldehyde |  | NA | – |
| Myrtenal |  | NA | – |
| Perillaldehyde |  | NA | – |
| Indole-3-carboxylaldehyde |  | NA | – |
| Octanal |  | 0.32 | 100 % (acid) |
| Decanal |  | 0.65 | 100 % (acid) |
| Phenylacetone |  | 0.37 | 100 % (benzylacetate) |

Regioisomeric excess (*RE*) is calculated as (product acid – product ester)/(product acid + product ester)

NA no detectable activity

Fig. 1 Reaction scheme of 2-phenylpropionaldehyde oxidation by BVMO4. The ester product in brackets was not observed but its hydrolysis product 1-phenylethanol was found

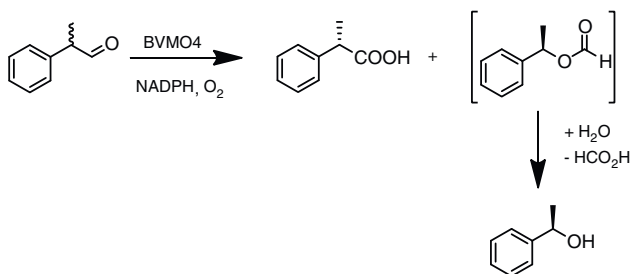


Table 4 Summary of the results from the oxidation of 2-phenylpropionaldehyde as performed in the screening and with four times excess of enzyme and substrate, respectively

| | Specific activity (U/mg protein) | <i>RE</i> (Regioisomeric excess) | <i>ee</i> 1-phenylethanol | <i>ee</i> 2-phenylpropionic acid |
|----------------------|----------------------------------|----------------------------------|---------------------------|----------------------------------|
| Screening conditions | 1.01 | 2 % (ester) | 7.2 % (<i>S</i>) | ND |
| 4X BVMO | 1.77 | 57 % (acid) | 28.5 % (<i>S</i>) | 40 % (<i>R</i>) |
| 4X substrate | 0.99 | 41 % (ester) | 7.8 % (<i>R</i>) | ND |

Regioisomeric excess (*RE*) is calculated as (product acid – product ester)/(product acid + product ester). One enzymatic unit is defined as the amount of enzyme necessary to oxidize one μmol of 2-phenylacetaldehyde in 1 min

ND not determined

according to the reaction conditions used (Table 4). Because of the low concentration of the acid product and the anionic charge of the carboxylic group, it was not possible to extract it efficiently, and its *ee* was measured only for the reaction in which the enzyme was in excess. In this case the (*R*)-enantiomer was formed with 40 % *ee*.

Furthermore our results show that BVMO4 was not inhibited when higher substrate concentrations were used; the reaction rate was similar to the one in screening conditions, but the specific activity of BVMO4 increased by almost two-fold when the enzyme concentration was increased four-fold (Table 4).

CHMO has been reported to oxidize bicyclo[3.2.0]hept-2-en-6-one with different enantioselectivity when the substrate concentration was varied [35]. A similar effect was observed for the oxidation of 2-phenylpropionaldehyde with BVMO4 and at present, it is not simple to rationalise the observations made here. Concerning the enantiocomplementarity of the acid and alcohol (ester) products. It may be assumed that the (*S*) substrate enantiomer preferentially undergoes the ‘normal’ Baeyer–Villiger oxidation whereas the other enantiomer is preferentially converted into the acid. This would, to some extent, explain the dependency of the *RE* on the substrate concentrations if different catalytic efficiencies (k_{cat}/K_m) are assumed for both enantiomers. The switch in *RE* upon increasing the biocatalyst concentration is more difficult to understand.

Aldehydes are known to covalently bind to enzymes by forming adducts with amino groups [36] and this could alter the properties of the enzyme, not least its activity and selectivity. In addition, possible allosteric effects could influence the enzyme’s stereochemical preferences. These assumptions are somewhat supported by the increased specific activity of the enzyme at increased enzyme concentrations. Currently, we have no information about the oligomerisation status of the enzyme at different concentrations and its possible influence on the stereopreference of the enzyme. Further investigations will be necessary to clarify this issue.

4 Conclusions

This paper has shown that *Dietzia* sp. BVMO4 catalyses oxidation of sulfide and aldehyde groups in a range of aliphatic and aromatic compounds. In all the cases that have been studied, when a chiral molecule is produced, the most abundant product was the (*R*)-enantiomer. Moreover, this enzyme has shown a unique feature compared to the previously published examples, i.e. the preferred oxidation product of aldehydes is the carboxylic acid rather than the ester.

This property is of special interest in case of the substrate 2-phenylpropionaldehyde, where the corresponding

carboxylic acid is the model compound for profens, a significant group of drug molecules. The (*S*)-enantiomer of profens is the bioactive molecule and the enzymatic synthesis of enantiopure profens is a hot topic in biocatalysis [21]. In our study, the regioselectivity of the reaction was increased in favour of the acid if the enzyme load is increased, though the enantioselectivity remained modest (40 % *ee*) and in favour of the non-bioactive molecule. Nevertheless, this is the first time that such reaction by a BVMO has been reported and in the future the regio- and enantio-selectivity can be improved by means of enzyme and reaction engineering. In addition, more aldehyde precursors of profens will be tested for oxidation by BVMO4 as this could represent an interesting alternative for the stereoselective synthesis of profens.

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Paper V

Enhancing the activity of a *Dietzia* sp. D5 Bayer-Villiger monooxygenase towards cyclohexanone by saturation mutagenesis

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Abstract

A recombinant Baeyer–Villiger monooxygenase, BVMO4 from *Dietzia* sp. D5 has been previously reported. The enzyme exhibited good thermostability and was active on a wide range of substrates. However, BVMO4 catalysed poorly the conversion of cyclohexanone to caprolactone. This work focuses on engineering BVMO4 to improve the conversion of cyclohexanone. A homology model structure was generated and used in combination with literature information on amino acids determining substrate specificity in other BVMOs to identify mutation “hotspots” that would influence substrate conversion. Site saturation mutagenesis was performed on 12 selected sites and 528 mutants were screened with expected coverage of about 98%. About one-fourth of the mutants screened exhibited more than 50% increase in cyclohexanone oxidation activity. Compared to the wild type BVMO, the best mutants, Y499I, Y499F and Y499L have shown about 11-12-fold increase for caprolactone production.

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Introduction

Caprolactone is one of the most useful lactones with a variety of important applications, the main ones being its use as a precursor to caprolactam, a monomer for the production of nylon, and biodegradable polymers. Polycaprolactone is an important product with useful biodegradable and biocompatible features [1]. In addition, caprolactone is used in the manufacture of specialty polyurethanes and as an additive for resins to improve their processing characteristics and their end use properties [2-3]. Polycaprolactone has been mixed with other natural polymers such as starch to lower cost and increase biodegradability [4], and moreover it can serve as a polymeric plasticizer to polyvinyl chloride [5].

The existing commercial production of caprolactone from cyclohexanone is chemical based and involves Baeyer-Villiger oxidation of the cyclohexanone using peracetic acid as oxidising agent. However, the high risk of explosion associated with the transport and storage of peracetic acid has led to the search for alternative methods of production [6]. Over the years, several catalysts such as transition metal catalysts, organocatalysts and biocatalysts have been considered as alternative tools to mediate the Baeyer-Villiger oxidation [6]. The enzyme catalysed production of caprolactone offers advantages over the chemical based process including safety, biodegradability and high specificity [7-8]. Among enzymes that have been considered for oxidation of cyclohexanone to caprolactone, Baeyer-Villiger monooxygenases (BVMOs) have attracted a lot of attention [9]. One well characterised BVMO that can oxidise cyclohexanone readily is the *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase (CHMO) [10]. This enzyme in vivo is involved in the oxidation pathway of cyclohexanol to adipic acid, therefore the oxidation of cyclohexanone by this BVMO is especially efficient. However, CHMO as well as other BVMOs suffer from some limitations [11], the major one being their instability. Hence the search for stable BVMOs is ongoing.

Recently, we have reported a stable BVMO (BVMO4) from a strain of *Dietzia* [12-13]. Although this enzyme efficiently oxidises a wide range of substrates, its activity on cyclohexanone was low. Thus, we aimed to improve the activity of this BVMO on cyclohexanone to enhance its caprolactone production efficiency. A number of mutational studies made so far have changed the substrate specificity of different BVMOs [14]. By combining the results of this studies with the information obtained from the homology model of BVMO4, potential “hotspots” sites for mutation have been selected. The mutation of these residues resulted in a remarkable increase in the conversion of cyclohexanone.

Materials and methods

Chemicals

Quikchange Lightning Multi Site-Directed Mutagenesis kit was purchased from Agilent Technologies (Santa Clara, USA). GeneJET plasmid purification kit was obtained from Thermo Scientific (Gothenburg, Sweden). Cyclohexanone and ϵ -caprolactone were acquired from Sigma-Aldrich (Stockholm, Sweden) and the other chemicals used for microbiological and molecular biology studies were purchased from Sigma-Aldrich, Calbiochem (Darmstadt, Germany) or Duchefa (Haarlem, The Netherlands).

Microorganisms, plasmids and primers

E. coli XL10-Gold ultracompetent and BL21-CodonPlus(DE3)-RP cells were purchased from Agilent Technologies (Santa Clara, USA) and the plasmid pET-22b(+) was purchased from Novagen (Darmstadt, Germany). The recombinant plasmid containing BVMO4 was prepared as previously described [13]. The mutagenic primers used in this study were synthesised at Thermo Scientific.

Homology modelling

Different approaches of comparative modelling and threading have been considered to generate the model. The top structural templates used to generate the model were phenylacetone monooxygenase from *Thermobifida fusca* (PAMO, PDB ID 1W4X), steroid monooxygenases from *Rhodococcus rhodochromus* (STMO, PDB ID 4AOS) and cyclohexanone monooxygenase from *Rhodococcus* sp. HI-31 (CHMO, PDB ID 3UCL) and the structural coordinates were retrieved from PDB. The percentage of sequence identity of these templates is summarised in Table 1. The quality of the model structure predicted was evaluated using ProSA and Procheck. The model has been visualised using Yasara (Yasara Biosciences, Wien, Austria) and PyMol (Schrödinger, Cambridge, USA). Multiple sequence alignments were prepared using T-Coffee [15] and visualised using CLC MainWorkbench (CLC Bio, Aarhus, Denmark).

Site saturation mutagenesis

Based on sequence and structural analysis and information gathered from previous studies, 12 sites (amino acids) have been selected for mutational studies. Saturation mutagenesis was performed with a restricted amino acid alphabet which has the advantage of generating smaller and, potentially, equally efficient libraries which are relatively easy to screen. The selected sites in this study were mutated with the codon NDT which encodes for 12 amino acids.

The amino acids obtained from the NDT alphabet are cysteine, aspartic acid, phenylalanine, glycine, histidine, isoleucine, leucine, asparagine, arginine, serine, valine and tyrosine.

The mutations were done using the primers containing the NDT codon (Table 2) and the Quikchange Lightning Multi Site-Directed Mutagenesis kit following the manufacturer's instructions. The PCR reaction was run according to the standard protocol with the addition of 1.5 μ l Quik solution to improve the PCR yield. After the completion of PCR, the template plasmid was digested using DpnI, and 4 μ l of the digested PCR mixture was used to transform ultracompetent *E. coli* XL10-Gold cells provided with the mutagenesis kit. The transformed cells were spread on Luria Bertani (LB) plates containing 100 μ g/ml ampicillin and incubated at 37 °C. After overnight incubation, the colonies (more than 200 colonies/plate) were collected from the plate using 2 ml LB and the plasmids were extracted using GeneJET plasmid purification kit according to the manufacturer's instructions (Fermentas). The plasmids were sequenced to check for the insertion of the desired mutations at GATC Biotech AG (Konstanz, Germany).

Library preparation and screening

Twelve single site mutant libraries were established by transforming mutated plasmids extracted from the *E. coli* XL10-Gold cells to chemically competent expression host cells, *E. coli* BL21-CodonPlus(DE3)-RP, and spread on plates containing ampicillin and chloramphenicol (34 μ g/ml). After overnight incubation, colonies were picked with a sterile loop and transferred to 400 μ l LB containing 100 μ g/ml ampicillin and 34 μ g/ml chloramphenicol in a sterile 96-well microtiter plate (polypropylene, squared bottom, 2.2 ml well volume). The plate was sealed with an oxygen permeable sterile film and incubated at 37 °C overnight with shaking at 250 rpm. Screening of the mutant BVMOs was performed by inoculating 380 μ l antibiotic containing LB in a sterile microtiter plate with 20 μ l of the overnight grown cultures. The cells from the remaining volume of the overnight cultures were recovered using 3-16PK centrifuge equipped with plate rotor (code 11240) at 4 °C and 4200 rpm. The cell pellet in each well was resuspended in 100 μ l sterile 10% (v/v) glycerol and transferred to a sterile standard microtiter plate and stored at -80 °C.

In each screening plate, two wells were dedicated to the negative control, *E. coli* cells harbouring pET-22b(+) plasmid without BVMO4 gene (wells A1 and A2), and another two wells (A3 and A4) were used for the positive control, *E. coli* BL21-CodonPlus(DE3)-RP cells expressing the wild type BVMO4. The other 44 wells were inoculated by colonies of *E. coli* BL21-CodonPlus(DE3)-RP expressing mutant BVMO4. The completeness of the

mutant library is calculated with GLUE-IT [16] and is estimated to be about 98%. The plates were incubated at 37 °C with shaking at 250 rpm until the optical density (OD₆₀₀) reached 0.6 (about 5 hours) and then the enzyme expression was initiated with addition of IPTG to a final concentration of 1 mM. At the time of IPTG addition, 2 mM cyclohexanone prepared in ethanol was also added and the incubation was extended at 15 °C.

After 18 hour of induction and biotransformation, the OD of the cell suspension in each well was measured by transferring 100 µl of the culture to a microtiter plate and reading the absorbance in Multiskan GO spectrophotometer (Thermo Scientific) at 600 nm. The remaining volume of the cultures was extracted by adding 400 µl of ethyl acetate to each well, vortexing the plate for 30 seconds to facilitate the extraction of the substrate (cyclohexanone) and the product (caprolactone) to the organic phase. The vortexed mixture was then centrifuged for 3 minutes to separate the phases. Samples from the organic phase were taken and transferred to GC vials and analysed.

Biotransformations

A limited number of mutants that exhibited an increase of more than 50% of the wild type BVMO activity on cyclohexanone were selected for a second round of screening to confirm that the observed increase in activity is reproducible. These biotransformations were performed in total reaction volume of 1 ml which contains 50 µl of the inoculum culture and 950 µl of LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol in 15 ml Falcon tube. The cultures were grown at 37 °C with shaking at 250 rpm. When the cell density (OD₆₀₀) was about 0.6, IPTG was added to a final concentration of 1 mM to induce the BVMO expression. At the time of IPTG induction, cyclohexanone was added to a final concentration of 5 mM. After 18 hours of incubation at 15 °C, the cultures were extracted by adding 500 µl ethyl acetate and vortexing. The two phases were separated by centrifugation, and the cyclohexanone and caprolactone contents of the organic phase was analysed by Gas Chromatography (GC) as described below.

As in the first round of screening, the negative control of *E. coli* cells carrying pET-22b(+) without BVMO4 gene and the positive control cells expressing the wild type BVMO4 were included in the biotransformation studies. All the biotransformation reactions were done as independent triplicates.

After the second round of screening, plasmids were extracted from mutants with improved activity and the BVMO4 genes were sequenced to identify the mutated residues.

GC analysis

Samples were analysed for cyclohexanone and caprolactone concentrations using Varian 430-GC gas chromatograph (Agilent Technologies, Santa Clara, USA). The separation of analytes was done using FactorFour VF-1ms 15 m × 0.25 mm (0.25 µm) column (Varian, Strathaven, UK) with 1 min hold at 50 °C; heating ramp from 50 °C to 225 °C at a rate of 25 °C/min; and 2 min hold at 225 °C (the injector and the detector temperature was maintained at 275 °C). Identification of the substrate and the product was done by comparison with retention time (rt) of commercial standards (cyclohexanone rt: 1.18 min; caprolactone rt: 2.59 min).

Results and discussion

Homology modelling

Based on sequence similarities, BVMOs can be categorised into different subgroups [17-18]. BVMO4 belongs to a subgroup of BVMOs that are closely related to CPDMO (cyclopentadecanone monooxygenase) of *Pseudomonas* sp. HI-70 known for oxidising large cyclic ketones (e.g. cyclopentadecanone) as well as the small substituted cyclic ketones and bicyclic ketones [19-20]. However, despite the sequence similarity, BVMO4 does not oxidise large cyclic ketones and cyclohexanone is oxidised with a relatively low efficiency compared to other substrates, such as phenylacetone or the closely related 2- and 3-methylcyclohexanone [13]. To improve the oxidation of cyclohexanone and understand the structure-function relation of the enzyme, it was necessary to make mutational studies. However, there is no determined structure for BVMO4 or for any other closely related BVMO. Thus, a homology model structure for BVMO4 was generated. The model structure would not only help in engineering *Dietzia* BVMO4 but can also contribute to shed some light on the structure of the CPDMO subgroup of BVMOs.

The maximum coverage in the threading alignment between the BVMO4 sequence and the closest template, 1W4X, Phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* is about 86% (Table 1). This is due to the fact that BVMO has a longer unaligned sequence at the N-terminal compared to PAMO, which is also reflected in the different overall length of the two proteins, that is 612 and 542 amino acids for BVMO4 and PAMO, respectively. The non-aligned section at the N-terminus, and to a lesser extent at the C-terminus region of the protein, is predicted as disordered by DISOPRED program. Therefore, the BVMO4 model structure was predicted using the residues of the enzyme between amino acid number 68 and 596.

As shown in Figure 1, superimposition of the models generated for BVMO4 revealed that except at the variable regions where the prediction varied depending on the parameters used, the rest of the structures are similar. The variable regions which correspond to the amino acid stretch between residue number 228-241, 319-334, 448-475 and 548-571, are part of loops and hence might be flexible.

The overall quality of the model is indicated by its Z-score value, which is shown as black dot in Figure 2 where the Z-scores of all the experimentally determined protein chains in current PDB vs number of residues in the proteins is plotted. The Z-score of the BVMO4 model without the disordered region of the protein is -9.24 while the entire BVMO4 (including the disordered part) has a Z-score value of -8.99. In general, the Z-score value of the generated model is within the range of scores typically found for native proteins of similar sizes. Moreover, the stereochemical quality of the model structure was checked using PROCHECK which provides an assessment of how normal or unusual is the geometry of the residues in the predicted model structure as compared to the stereochemical parameters derived from high-resolution structures. The Ramachandran plot (Figure 3) shows that only 0.4% of the residues are in the disallowed region which suggests the geometry of the residues in BVMO4 model seem to fall within the thermodynamically favored geometries.

Since residues of the active site region of BVMO4 are of prime importance in engineering its catalytic property, the active site area of BVMO4 was analysed and compared to that of PAMO (1W4X) (Figure 4), in addition to identifying the cofactor binding residues of BVMO4 which are listed in Table 3.

Identification of “hotspots” for mutagenesis

With the help of the homology model and information that has been gathered from available reports on mutations of BVMOs, 12 sites were chosen and targeted for mutation. The sites selected and the corresponding residues in other BVMOs mutated by other researchers are shown in Table 4 and Figure 5.

The residues I120, D351, Q352, A498, Y499, Y500, V501, F503, V504 were selected because of their proximity to the active site. Generally, residues close to the active site were picked if there has been favourable report on mutational studies and are not highly conserved. For instance, the conserved residues R394, known as “catalytic arginine”, has an important structural/functional role and its mutation would severely impair the enzyme function. A similar observation is valid for other residues involved in binding of the cofactors, such as Y113 or D119. The other group of selected residues known as the

“second sphere” residues are not as close as the first group of residues to the active site. The reason behind this selection is the positive influence reported from the mutation of residues at the sites. This group of residues include G143, S144 and T497. T497 was especially selected because it is the first residue of a stretch of amino acids starting from A498 up to F503 (according to BVMO4 sequence numbering) that has been highlighted in several reports to mainly influence the reactivity of BVMOs, [21-23].

Mutant libraries and screening

Twelve libraries were established and a total of 528 mutants (44 mutants for each library) were screened for their ability to convert cyclohexanone to caprolactone. The first round of screening revealed that about 120 mutants exhibited more than a 50% increase on cyclohexanone oxidation activity.

The saturation mutagenesis libraries of the residues T497, A498, Y499, V501 and V504 contained high number of mutants with improved cyclohexanone conversion: 30% or more clones of each library showed at least 50% improvement over the wild type. This is in agreement with previous reports which have shown that mutation of these sites influenced substrate specificity of BVMOs. Another major improvement of cyclohexanone activity has been observed in I120 and Y500 mutant libraries. In particular, the I120 residue interacts with the flavin ring, therefore mutation at this site is expected to influence the conformation of the active site.

Screening of library F503 did not result in significantly improved variants. This is in contrast to what has been reported for PAMO where the corresponding residue M446 mutation to glycine significantly improved PAMO activity [23-24]. The libraries of G143 and S144 did not show significantly improved mutants however the enzyme activity of the mutants in the library was not compromised. Unlike the other libraries, there was no improved mutant identified in the libraries of D351 and Q352.

The best mutants identified in each library were selected and re-screened for biotransformation of cyclohexanone to further confirm the improved feature (Figure 6). Among the best mutants Y499I, Y499L and Y499F have shown the highest increase in cyclohexanone oxidation activity which is in the range of 11-12 fold. These three best mutants are followed by I120V, Y500G and Y500F which are more than five times more active on cyclohexanone than the wild type BVMO4. It is important to make an in depth analysis of the model structure and substrate docking studies to explain why these mutations resulted in enhanced activity. One interesting observation is that most of the residues that exhibited significant improvement of cyclohexanone activity are hydrophobic amino acids. The change towards more hydrophobic residues is

expected to improve accommodation of the hydrophobic substrate and hence lead to an improved enzyme activity.

Conclusion

The results obtained in this study revealed that the strategy used to select and mutate the residues was successful. About 20% of the total mutants screened exhibited at least 50% increase in the cyclohexanone oxidation activity. A significant improvement, up to 12-fold increase, has been achieved by a handful of mutants. Such an improvement is among the highest reported for BVMO mutation studies aimed at improving activities [25-28]. As the amino acid stretch from positions 497 to 504 seems to play a critical role in the enzyme activity and substrate specificity, these sites can be used as a starting point for engineering the catalytic properties of BVMOs. Nevertheless, other sites outside of this stretch, such as I120, can also improve considerably the conversion. In addition, it is possible that sites not targeted in this study could also play an important role for BVMO4 reactivity and more powerful mutagenic techniques, such as directed evolution, should be used to address such question.

An iterative mutagenesis approach may further enhance the activity of the best mutants obtained in this study. Finally, characterization of the best BVMO4 mutants is necessary, in particular the stability has to be determined and compared to the wild type enzyme.

Acknowledgments

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Table 1: Sequence identity and coverage of templates used for the BVMO4 structure prediction. Identity 1 is the percentage sequence identity of the templates in the threading aligned region with the query sequence. Identity 2 is the percentage sequence identity of the whole template chains with query sequence. Coverage refers to the coverage of the threading alignment and is equal to the number of aligned residues divided by the length of query protein

| PDB ID | Identity 1 | Identity 2 | Coverage |
|--------|------------|------------|----------|
| 1W4X | 0.3 | 0.28 | 0.86 |
| 4A0S | 0.29 | 0.28 | 0.84 |
| 3UCL | 0.27 | 0.26 | 0.82 |

Table 2: Mutagenic primers used in the saturation mutagenesis of BVMO4

| Mutated residues | Primer sequence (5'-3') |
|------------------|--|
| I120 | catgtagatgtgcgactcAHNatcgactgcacgcc |
| G143 | ccagcgctacgcggatNDTtccgagatcttcgagc |
| S144 | gcgtgctcgaagatctcAHNaccatccgctagcgc |
| D351 | gaactccccgaggggaNDTcaggcctggtcgcg |
| Q352 | aactccccgagggggacNDTgccctggtcgcg |
| T497 | gaagttgacgtagtaggcAHNctgcgacatccgagc |
| A498 | gacgaagttgacgtagtaAHNngtctgcgacatccc |
| Y499 | catgtagacgaagttgacgtaAHNggcggctctgcgacatcc |
| Y500 | tgtcgagaccgcctacNDTgtcaacttcgtctacatgc |
| V501 | cgagcatgtagacgaagttAHNgtagtaggcggctctgcg |
| F503 | accgcctactacgtcaacNDTgtctacatgctcgaccgc |
| V504 | cttgcggtcgagcatgtaAHNgaagttgacgtagtaggc |

Table 3: Residues of the cofactor binding site of BVMO4. In the first group are those that bind FAD, in the second column those binding NADPH.

| <u>FAD binding</u> | <u>NADPH binding</u> |
|--------------------|----------------------|
| 74 | L 113 Y |
| 75 | G 117 Q |
| 77 | G 119 D |
| 78 | F 203 L |
| 79 | G 252 G |
| 99 | E 253 T |
| 100 | Y 254 G |
| 106 | G 255 A |
| 107 | T 256 T |
| 108 | W 259 Q |
| 110 | W 276 R |
| 111 | N 277 T |
| 118 | C 279 S |
| 119 | D 442 A |
| 120 | I 443 T |
| 125 | Y 444 G |
| 167 | T |
| 168 | R |
| 169 | A |
| 199 | S |
| 200 | N |
| 201 | G |
| 202 | A |
| 203 | L |
| 457 | Y |
| 503 | F |
| 507 | L |

Table 4: Summary of the mutants and their properties with information from previous literature reports.

| Residue | Reported mutations at corresponding site | Location and effect of the mutation | Ref. |
|---------|--|---|--------------|
| I120 | I67T (PAMO) | Located within 8 Å from the flavin ring. | [25] |
| | V72I (STMO) | Inversion of stereoselectivity of thioanisole sulfoxide (PAMO). Increase of k_{cat}/K_M for phenylacetone (STMO). | [27] |
| G143 | Q93N (PAMO) | Located in the N-terminal region of an α -helix which interacts with loops at 18 Å from the FAD. The combined mutant at position 93 and 94 accepted the substrates (2-substituted cyclohexanones) while WT PAMO did not. Only simultaneous mutation of position 93 and 94 is effective, not the single sites taken separately. | [29] |
| S144 | P94D (PAMO) | See residue G143. | [29] |
| D351 | F277S (CHMO) | No corresponding residues to these in BVMO4, according to the sequence alignment it is between position 351 and 352 (BVMO4) | [30] |
| | P286A, R, F (PAMO) V291A (STMO) | Inversion of the enantioselectivity of the sulfoxidation (CHMO). Within 5 Å from the FAD, it influenced enantioselectivity and conversion of 4-substituted cyclohexanone in combination with other mutations (PAMO). Neutral mutation in STMO. | [26] [27] |
| Q352 | | See residue D351. | |

| | | | |
|------|---|--|---|
| T497 | P440F, L, I, N, H, W, Y (PAMO) | “Second sphere” residue not directly in contact with the binding pocket. P is conserved in many BVMOs but not BVMO4. P440 library mutants oxidise substrates not accepted by the wild type (2-substituted cyclohexanones). | [31]. |
| A498 | S441P,D (PAMO) S441A (PAMO) | Within 5 Å from the active site. It can influence enzyme reactivity in combination with other sites. | [25] [26] |
| Y499 | A442G (PAMO) G499S (CPMO) | Within 5 Å from the active site. Changes in PAMO reactivity against sulfides. In combination with F450, change in substrate (4-substituted cyclohexanone) conversion of CPMO. | [25- 26] [32] |
| Y500 | F432I (CHMO) F432S (CHMO) L443D, A, F (PAMO) L443F (PAMO) F450Y, I, C (CPMO) | Within 5 Å from the active site. Mutation in combination with T433 changed regiospecificity of 4-substituted cyclohexanones oxidation. Also mutation of the residue alone changed speteroselectivity. It is close to the substrate binding area and it was investigated its influence on substrate binding using molecular dynamic simulations (CHMO). Its mutation changes substrate specificity for sulfides and 4-substituted cyclohexanones (PAMO). See Y499 for CPMO mutation. | [33] [34] [30] [35] [25- 26] [28] [32] |
| V501 | S444C (PAMO) S444A (PAMO) | Within 8 Å from the active site. Improved reactivity against bicycloheptanone and 4-substituted cyclohexanones in combination with other residues. | [25- 26] |
| F503 | M446G (PAMO) | Binds the FAD ring with the backbone. It dramatically changes the specificity of PAMO for a number of substrates (PAMO). | [23, 36] |

| | | | |
|------|--------------|---|------|
| V504 | L447P (PAMO) | Within 8 Å from the active site. It changed the specificity of the enzyme mostly when in combination with other mutations. | [25] |
|------|--------------|---|------|

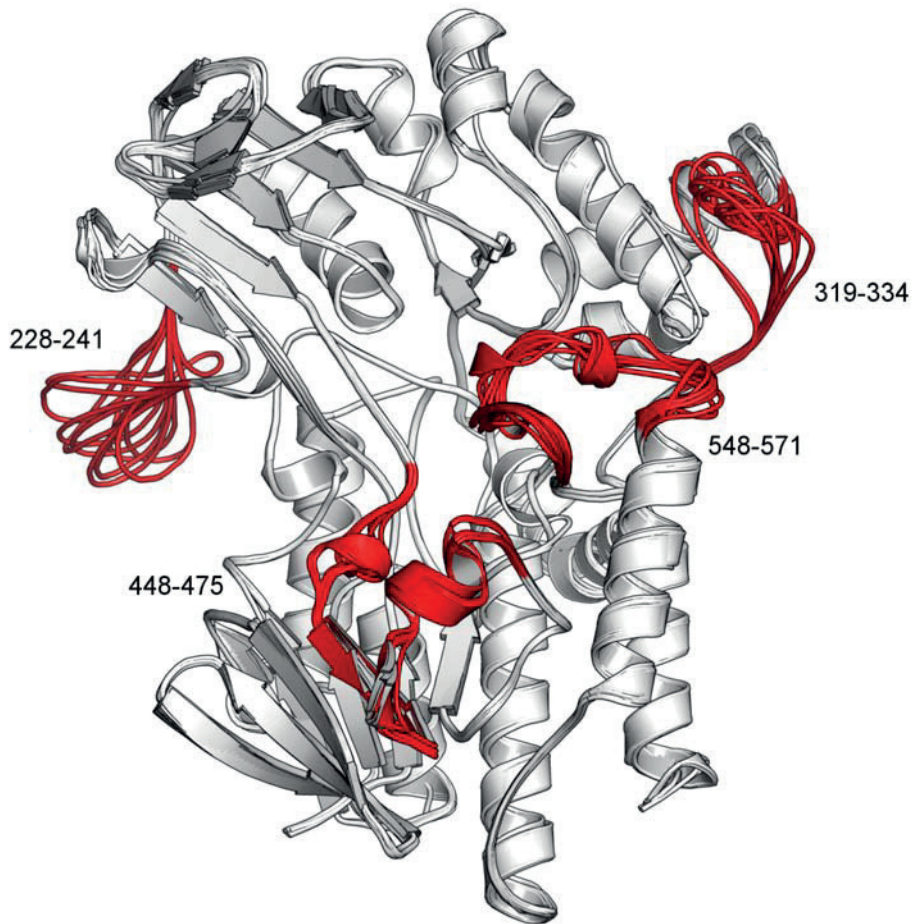


Figure 1: The predicted model structure of BVMO4. The image represents the superimposed predictions of BVMO4 structures and the variable regions of the protein structure are highlighted in red.

Z-Score = -9.24 (without the termini part - left side)

Z-Score = -8.99 (full model - right side)

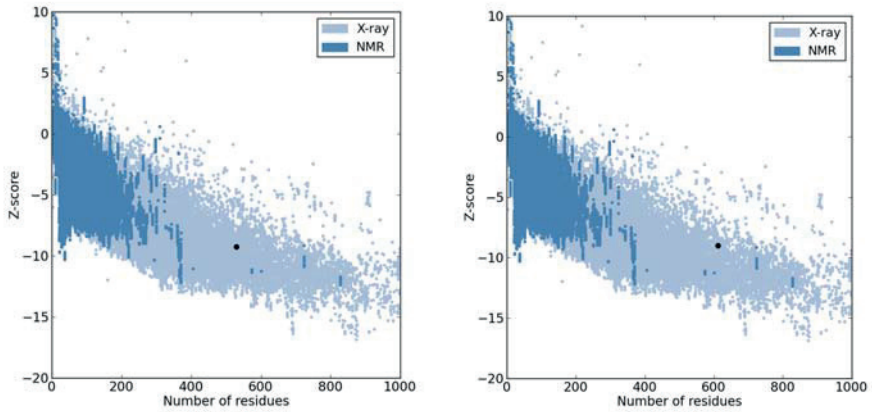


Figure 2: Z-score value of the BVMO4 model in the presence (right) and absence (left) of the disordered C- and N-terminal regions.

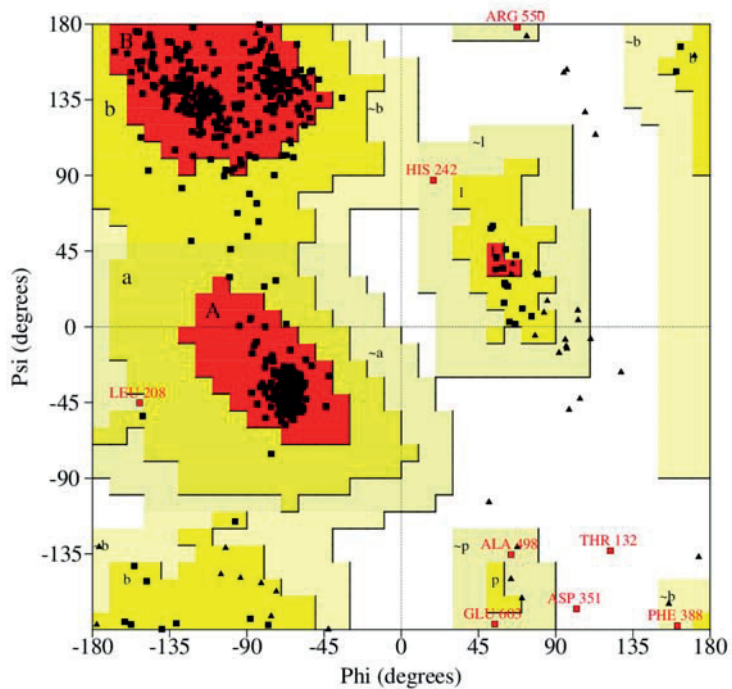


Figure 3: Ramachandran plot of the BVMO4 model structure

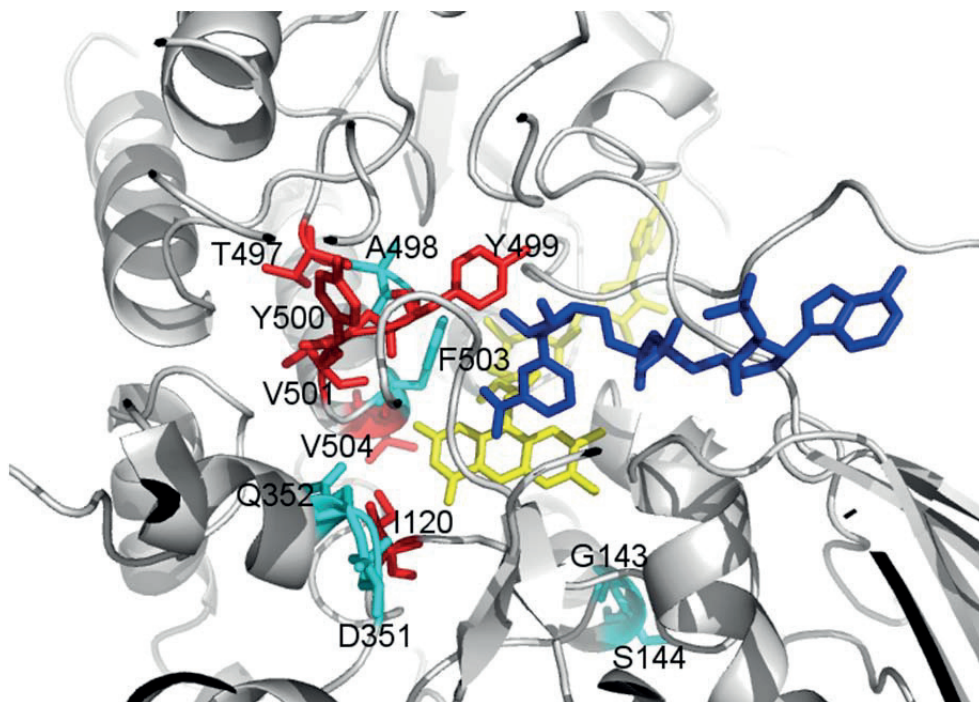


Figure 5: Snapshot of the protein cavity with the mutated residues. In red are the residues which showed an increase of at least 2-fold, in cyan are the other residues mutated. In yellow is FAD, in blue NADPH and in white is the whole protein backbone.

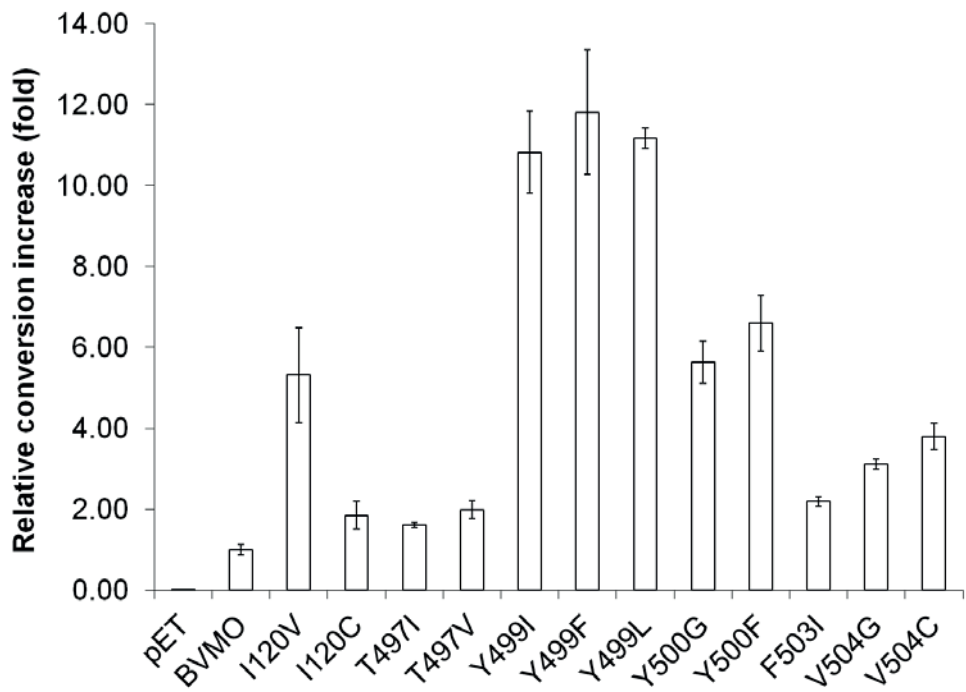


Figure 6: Relative activities of the best BVMO4 mutants on cyclohexanone.

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