Laboratory of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

Discovery of putative monooxygenases from *Rhodococcus* sp. RHA1

Cloning and substrate identification of putative Baeyer-Villiger monooxygenases

Michael Verhoeven (1218298)

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Abstract

The Baeyer-Villiger reaction inserts an oxygen atom into specific carbon-carbon bonds. The reaction is able to form compounds which form the basis for industrially interesting products. Baeyer-Villiger monooxygenases (BVMOs) are enzymes able to catalyze this specific reaction, and several other oxidation reactions (e.g. sulfoxidation). The BVMOs provide a more attractive alternative to traditional chemistry for performing the reaction. To broaden the range of recognized substrates by available BVMOs, the genome of *Rhodococcus* jostii RHA1 was screened with two sequences of genes with known function. The cloning of four genes with good (>40%) sequence similarity resulted in the expression and purification of a LimB homolog. The enzyme was found to catalyze sulfoxidation reactions with high enanthioselectivity.

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Introduction

The Baeyer-Villiger reaction allows breaking of specific carbon-carbon bonds with the incorporation of an oxygen atom, thereby able to form compounds which form the basis for industrially interesting products. The reaction was discovered by Adolf Baeyer and Victor Villiger in 1899, and uses peracid or hydrogen peroxide in combination with rearrangement of the bonds to incorporate an oxygen atom, as shown in figure 1 [Mihovilovic, 2002].

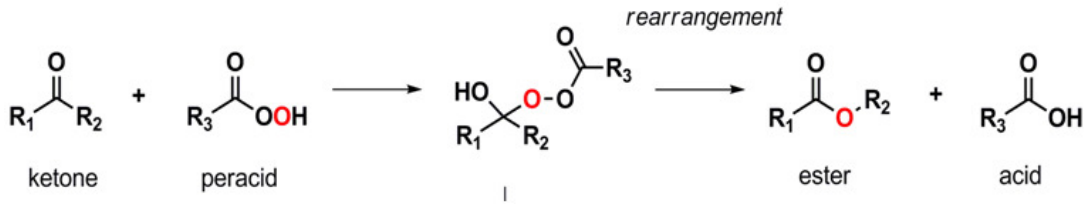

Figure 1: The Baeyer-Villiger reaction catalyzed by peroxide acids. The red oxygen atom is transferred from the peroxide acid to the ketone, and incorporated to form an ester.

The conditions under which the reaction is performed chemically requires extensive safety precautions, due to the use of very unstable peracids. In addition, the reaction leads to product formation with low selectivity, thereby producing a considerable amount of waste stream. Baeyer-Villiger monooxygenases (BVMO) form a class of enzymes able to perform the Baeyer-Villiger reaction, thereby offering high selectivity and milder reaction conditions.

A large number of BVMOs have been reported over the last several years [van Berkel, 2006]. BVMOs are able to incorporate an oxygen atom in organic molecules with the aid of a flavin cofactor, instead of peracids, as shown in figure 2.

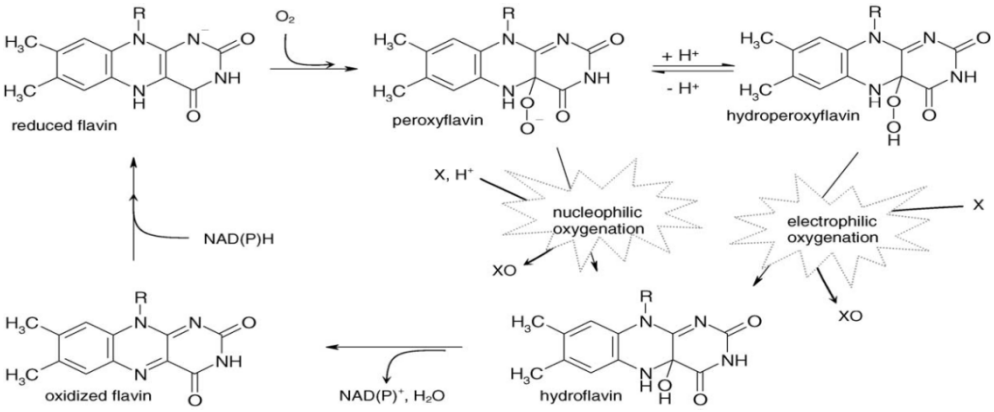

Figure 2:Proposed mechanism of (hydro)peroxyflavin formation and regeneration of the flavin cofactor [van Berkel, 2006]. The balance between the peroxy- and hydroperoxyflavin state show the ability of BVMOs to perform both nucleophilic and electrophilic oxygenation reactions.

BVMOs can catalyze both nucleophilic and electrophilic oxygenation reactions, depending on the state of the flavin cofactor being in the peroxy or hydroperoxy form. The use of flavin as cofactor, and the orientation of substrate in the active center, result in high regio- and enantioselectivity, and is unrivaled by traditional chemical reactions. However, the restricted available substrate range, due to a limited number of known BVMOs, still leaves room for improvement. To increase the amount of recognized substrates by available BVMOs, redesign of existing enzymes or discovery of new BVMO enzymes is necessary.

BVMOs can be divided into at least two classes based on composition and use of cofactor. The Type I BVMOs are made of one polypeptide chain, have FAD as a bound cofactor, and dependent on NADPH for activity. Type II BVMOs consist of two separate subunits, use FMN as cofactor, and depend on NADH for activity [Pazmiño, 2008]. In comparison to Type I BVMO's, Type II BVMO's have been reported to a far lesser extent [Kamerbeek, 2003]. The difference is mainly due to the relative uncomplicated system of one polypeptide chain in the case of Type I BVMOs and typical solubility of the enzymes [Pazmiño, 2008].

In order to find new BVMOs and widen the available substrate range for reactions, three main strategies can be applied. First, isolating and cultivating organisms followed by screening for BVMO activity has widely been used in the past, but fails to pick up activity of non-cultivatable organisms. Second, isolation of large quantities of DNA into metagenomic libraries tackles the problem of non-cultivatable organisms by collecting their DNA in large plasmids. Downside to this method is the large amount of work involved in constructing the libraries and screening all parts for activity. The third method makes use of the increasing number of deposited sequenced genomes, facilitating in silico search for relevant genes. The method is applied here, with the use of the genome of *Rhodococcus* sp. RHA1, providing the opportunity to rapidly identify interesting genes.

Rhodococcus jostii RHA1 is an actinomycete, a Gram-positive bacteria originating from lindane contaminated soil. These bacteria are capable of using a wide range of substrates for growth, and are known for their capability to degrade polychlorinated biphenyls (PCBs) [McLeod, 2006]. The ability to degrade a wide range of substrates makes *R.* jostii RHA1 interesting for further investigation into the different types of enzymes and the reactions they can perform [Larkin, 2005]. Exploring the genome with a sequence motif identified in BVMO sequences, led to an indication of at least 20 putative Type I BVMO genes. Current studies

are being performed to investigate these genes for their function. An alternative approach is to screen the genome with sequences of known (BVMO) genes which may have interesting substrates or functions.

The ability to use n-alkanes as a substrate by enzymes and incorporate an oxygen atom is known for the range of alkanes from C_1 to C_{44}. Most enzymes are capable of utilizing substrates in the range of C_1 to C_{16}, and only few are capable of utilizing carbon chains of C_{17+}. Novel enzymes capable of using such long chains could have a potential application in petrochemical industry or environmental cleanup. Long chain alkane monooxygenase, LadA, is able to incorporate an oxygen atom in carbon chains ranging from C_{15} to C_{36} with the use of a flavin cofactor [Li, 2008]. LadA was discovered in *Geobacillus thermodenitrificans* NG80-2, and turns the long chain alkanes into the corresponding primary alcohols [Feng, 2007].

Limonene monooxygenase (LimB) from *Rhodococcus erythropolis* DCL14 is considered to be a model Type II BVMO able to use limonene as substrate [van de Werf, 1999]. To increase the available Type II BVMOs and possibly increase the range of recognized substrates the sequence of LimB was used to screen for homologs. Although LadA does not perform a Baeyer-Villiger reaction, the hydroxylation of carbon chains makes screening for homologs interesting.

Screening of the genome of *Rhodococcus* jostii RHA1 with above sequences of LadA and LimB led to the discovery of one LadA homolog and three LimB homologs with good sequence similarity (>40%). All four genes were cloned into pBAD expression vectors under the control of an araBad promoter. Purification of one LimB homolog led to substrate screening assays for possible BVMO activity. Product identification resulted in several possible substrates, showing that the enzyme is capable of performing enantioselective sulfoxidation reactions.

Materials and Methods

Materials

Expression vectors pBAD and pCRE3C were present at the lab as PAMO constructs from previous studies. Primers were obtained from Sigma-Genosys (Sigma-Aldrich). All chemicals used were available at the lab. Constructs of all genes were sequenced at GATC Biotech (Germany).

Strain and growth conditions

Escherichia coli strain TOP10 was made available as competent cells in 100 µL volumes. Cells were grown in Luria-Bertani (LB) medium[1] or on LB plates (1.5% agar (wt/v)) containing 100 µg/ml of ampicillin (Amp) antibiotics during transformation and enzyme expression. Cells were grown at 17˚C, 24˚C, 30˚C, or 37˚C during enzyme expression, depending on the optimal temperature for maximum expression, with constant shaking (200 rpm). The expression of enzyme was induced with a L-arabinose concentration of 0.02%.

Genes from Rhodococcus sp. RHA1

BLAST searches were performed with the BLAST tool on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the *Rhodococcus* sp. RHA1 (taxid: 101510) as target organism. The template sequences used to find possible monooxygenase sequences were limonene monooxygenase [*Rhodococcus erythropolis*, GeneID: 4968539] and alkane monooxygenase [*Geobacillus thermodenitrificans* NG80-2, GeneID: 12054950].

PCR

The *mox1*, *mox2*, *mox3*, and *mox4* genes were amplified by PCR from *Rhodococcus* sp. RHA1 genomic DNA, including the three genomic plasmids. The primers were designed with NdeI and HindIII restriction sites for cloning in the pBAD vector. For the LadA homolog gene, a second forward primer was designed, with PvuII restriction site for cloning in the pCRE3C vector, see table 1.

[1] LB medium consists of 10 g /L bacto tryptone, 5 g /L yeast extract and 5 g /L NaCl.

Table 1: Primers designed for amplification of the genes having good sequence similarity on the genome of *Rhodococcus* jostii RHA1.

Gene name		Primers (5'-3')	Restriction Site in Blue
Mox1 (pBAD)	fwd	AGGAGGAATTACATATGACCCGGCAGATCCG	NdeI
,,	rev	GTTCGGGCCCAAGCTTTTAGTTGTTCGTGTACGCGC	HindIII
Mox2 (pBAD)	fwd	AGGAGGAATTACATATGTCCGAATCCAACTACCC	NdeI
,,	rev	GTTCGGGCCCAAGCTTTTAGTTGACCGATACCGGC	HindIII
Mox3 (pBAD)	fwd	AGGAGGAATTACATATGTTCTCTTGTGCAAATATTCC	NdeI
,,	rev	GTTCGGGCCCAAGCTTTTAGGCCCTCCCAGCC	HindIII
Mox4 (pBAD)	fwd	AGGAGGAATTACATATGGAACCGGAAGCTGG	NdeI
,,	rev	GTTCGGGCCCAAGCTTTTACCCTGCGCTCTTCG	HindIII
Mox1 (pCRE3C)	fwd	ACTCGAGATCTGGCAGCTGGTATGACCCGGCAGATCCG	PvuII

The PCR reaction was performed according to the following program: initial denaturation at 98˚C for 30 sec., followed by 35 cycles of 40 sec. at 98˚C, 30 sec. at 62˚C -> 52˚C (Touch Down Annealing) and 55 sec. at 72˚C.

In-Phusion cloning

PCR products were purified using the standard procedure of the NucleoSpin®Extract II kit, followed by pretreatment with cloning enhancer and incubation at 37˚C for 20 minutes and 80˚C for 15 minutes. The PCR products were mixed with either digested pBAD or pCRE3C vector in a 2:1 molar ratio, and subsequently added to freeze dried In-phusion cloning enzyme (Clontech), and incubated at 37˚C for 15 minutes and 50˚C for 15 minutes. The solutions were diluted with TE-buffer[2] (pH 8.0) and stored on ice. The cloned *mox* genes were confirmed by DNA sequencing at GATC Biotech (Germany).

Transformation

E. coli TOP10 cells were mixed with 5 µl plasmid DNA and incubated on ice for 30 minutes. The cells were subjected to a heat-shock of 90 sec. at 42˚C, in a preheated heat block, followed by incubation on ice for 2 minutes. After addition of 0.9 ml LB medium, the cells were incubated at 37˚C for 1 hour, and 100-200 µl was transferred to LB agar plates containing 100 µg/ml ampicillin. The agar plates were stored at 30˚C over night to let colonies grow.

Expression conditions

Expression of all four enzymes was tested at varying temperatures and L-arabinose concentrations during growth of the cells. The temperature was varied between 17°C, 24°C,

[2] TE-buffer consists of 10 mM Tris/HCl, pH 8.0 and 1 mM EDTA.

30°C, and 37°C and concentrations of L-arabinose of 0%, 0.0002%, 0.02% and 2% (wt/v) were used. The amount of protein was checked with the use of SDS-PAGE gel electrophoresis (see below), by resuspending the pellet from one ml of culture in 500 µl Tris/HCl buffer (pH 7.5). The resuspended cells were disrupted by sonification (4 times 5 seconds, 15 seconds interval, standard tip) and subsequently centrifuged for 5 minutes at full speed. Soluble protein should be in the supernatant fraction after centrifugation. Apart from Tris/HCl buffer (pH 7.5), three additional buffers were used to test the solubility of the Mox1 (pBAD and pCRE3C) enzyme, namely LDAO-buffer[3], Brij 35-buffer[4] and DMP-buffer[5]. These buffers were used to resuspend the pellets before sonification.

Inclusion bodies

In the case of Mox1 (pBAD and pCRE3C) a Mini-scale Protein Extraction was performed to check for inclusion body formation. Cells from 1 ml of culture were pelleted and redissolved in 150 µl B-per II® reagent by vortex for 2 minutes. The solution was centrifuged for 5 minutes at full speed to separate the soluble and insoluble proteins. The pellet was resuspended in fresh B-per II® reagent and centrifuged for 10 minutes at 5000 rpm. The pellet was washed with 2x 500 µl Tris/HCl buffer (pH 7.5), redissolved in 500 µl 50 mM Tris/HCl buffer (pH 7.5) and centrifuged for 5 minutes at full speed.

Overexpression

E. coli TOP10 cells, containing the plasmid pBAD-mox-2, were used to inoculate 5 ml LB medium containing 100 µg/ml ampicillin and grown over night at 30˚C. The preculture was added to 500 ml TB medium[6] containing 100 µg/ml ampicillin and 0.02% L-arabinose. After an OD_{600} of 1.8 was reached, cells were harvested by centrifugation in a J10 rotor 6000 rpm for 15 minutes at 4˚C. Pellets re-suspended in 50 ml Tris/HCl pH 7.5 and centrifuged in small centrifuge 30 min. 4000 rpm at 4˚C.

[3] LDAO-buffer consists of 50 mM Tris/HCl pH 7.5, 10% glycerol, 0.5 mM DTT, and 1% lauryldimethylamine-n-oxide.

[4] Brij-35 consists of 50 mM Tris/HCl pH 7.5, 10% glycerol, 0.5 mM DTT, and 1% polyethylene glycoldodecyl ether.

[5] DMP-buffer consists of 50 mM Tris/HCl pH 7.5, 10% glycerol, 0.5 mM DTT, and 2% n-dodecyl-β-D-maltopyranoside.

[6] TB medium consists of 12 g /L bacto tryptone, 24 g /L yeast extract, 4 ml glycerol, 2.32 g/ L KH_2PO_4, and 12.54 g/ L K_2HPO_4.

Purification

Collected cells were washed twice with 50 mM Tris/HCl buffer (pH 7.5), and re-suspended in 20 ml of 50 mM Tris/HCl buffer (pH 7.5). Cells were disrupted by sonification for 7.5 minutes with 2 sec. on – 2 sec. off cycles at 0.8˚C. Suspension was subsequently centrifuged in a JA-17 rotor for 15 min at 4˚C. The enzyme in the cell extract was purified with an Äkta – DEAE sepharose column, using 50 mM Tris/HCL pH 7.5 buffer and 50 mM Tris/HCl pH 7.5, 2.0 M KCl buffer to create a linear gradient. The purified enzyme was concentrated and desalted with an ultrafiltration unit (Amicon) to a final volume of 10 ml.

SDS-PAGE

Acryl amide gels (12.5%) were prepared with the use of a standard assembly unit from Bio-Rad Laboratories. The running gel was prepared by mixing 2 ml acryl amide, 2.7 ml dH_2O, 1.65 ml running buffer, 30 µl ammonium persulfate, and 4 µl TEMED. Stacking gel was prepared by mixing 0.32 ml acryl amide, 1.5 ml dH2O, 0.62 ml stacking buffer, 15 µl ammonium persulfate and 4 µl TEMED.

Substrate screening

The in vitro substrate screening assay was based on a previously described assay (Van Hellmond, 2007), using either FMN or FAD, NADH, and purified Mox2 solution. Standard reaction solution contained: 200 µM NADH, 25 µM FMN, 1 mM substrate, and 10 µl Mox2 solution with a total volume of 1 ml. Reactions were quenched by addition of an equal amount of tert-buthyl-methyl-ether with dioxane as internal standard, and subsequent extraction by vortex for one minute. The organic layer was dried with $MgSO_4$ and stored for later analysis.

UV spectroscopy

In order to optimize the substrate screening assay and determine the substrates to be tested, multiple tests were performed using a Lambda Bio 40 UV/VIS spectrophotometer. The total reaction volume of each measurement was 100 µl by using a black coated 100 µl quartz cuvette. Standard reaction solutions as described above were used to test the state of FMN (reduced or oxidative state).

Gas chromatography

In order to quantify the degree of conversion, product formation was measured using a nonchiral HP1 capillary GC column (30 m by 0.25 mm) with tert-butyl-methyl-ether as solvent and dioxane as internal standard. The following temperature program was used: 100°C to 200°C with 10°C/min and hold 5 min at 200°C. For sulfoxidation reactions, samples were analyzed on a Chiraldex GTA capillary column (30 m by 0.25 mm) with the following temperature program: 35°C to 200°C with 15°C/min and then 5 min at 200°C.

Data analysis

GC measurements were analyzed by calculating the enantiomeric excess of the sulfoxides. The enantiomeric excess (ee) can be calculated by using equation 1, in which the R and S represent the two different enantiomers.

$$ee = \frac{R - S}{R + S} \times 100\% \qquad (1)$$

Conversion of substrate was determined by taking the area under the substrate and product peak in consideration. The areas of the substrate (A) and product (P) peak were divided by the area of the internal standard, resulting in A_S and P_S, respectively. The conversion was calculated according to equation 2.

$$conversion = \frac{A_S}{A_S + P_S} \times 100\% \qquad (2)$$

Results

Search results

The genome sequence of *Rhodococcus* sp. RHA1 (taxid: 101510), as deposited on the NCBI website, was used to identify novel putative Baeyer-Villiger monooxygenase (BVMO) genes. Protein BLAST's with the sequence of a long chain alkane monooxygenase (LadA) from *Geobacillus thermodenitrificans* NG80-2, as well as the sequence of a limonene monooxygenase (LimB) from *Rhodococcus erythropolis*, were performed. Four genes with >40% sequence identity, see table 2, were chosen for further investigation.

Table 2: Gene information of both known BVMO genes, LadA and LimB, together with the genes having good sequence similarity on the genome of *Rhodococcus* jostii RHA1.

Gene name		**Organism**	**Gene Information**					
			GeneID	*Locus tag*	*Location*	*Length (exp. wt)*	*Identity*	*Gap*
Alkane monooxygenase	LadA	*Geobacillus thermodenitri-ficans* NG80-2	4968539		pLW1071	440 a.a.		
Monooxygenase	Mox1	*Rhodococcus* jostii RHA1	4220434	RHA1_ro02882	Genome	471 a.a. (52 kDa)	51%	4%
Gene name		**Organism**	**Gene Information**					
			GeneID	*Locus tag*	*Location*	*Length (exp. wt)*	*Identity*	*Gap*
Limonene monooxygenase	LimB	*Rhodococcus erythropolis*	12054950		Genome	387 a.a.		
Limonene monooxygenase	Mox2	*Rhodococcus* jostii RHA1	4225509	RHA1_ro09085	pRHL1	389 a.a. (43 kDa)	49%	1%
Limonene-1,2-monooxygenase	Mox3	*Rhodococcus* jostii RHA1	4226479	RHA1_ro11046	pRHL3	420 a.a. (47 kDa)	46%	1%
Monooxygenase	Mox4	*Rhodococcus* jostii RHA1	4219425	RHA1_ro00392	Genome	407 a.a. (45 kDa)	43%	3%

Sequence alignment (see appendix A) showed LimB homolog Mox2, Mox3 and Mox4 had a comparable sequence identity compared to each other, ranging from 40% to 50% sequence identity, indicating they are not isoforms of one gene, and can be considered as separate genes.

The genes encode for putative monooxygenases, with in the case of *mox2* possible involvement in several KEGG degradation pathways, as indicated on the NCBI website. Further indication of monooxygenase activity can be supported by flanking genes on the genome. In the case of *mox2* the gene is flanked by a flavin reductase in opposite direction (GeneID 4225511, RHA1_ro09087). In the case of *mox3* the gene is flanked by an oxidoreductase (GeneID 4226480, RHA1_ro11047) and limC3 dichlorophenolindophenol-dependent carveol dehydrogenase (GeneID 426477, RHA1_ro11044). In the case of *mox4* the

gene is flanked by a carveol dehydrogenase (GeneID 4219424, RHA1_ro00391). For these three genes the search was performed by using a known limonene utilizing enzyme, which could have carveol as a product. The appearance of carveol dehydrogenase could indicate involvement of the genes in a degradation pathway.

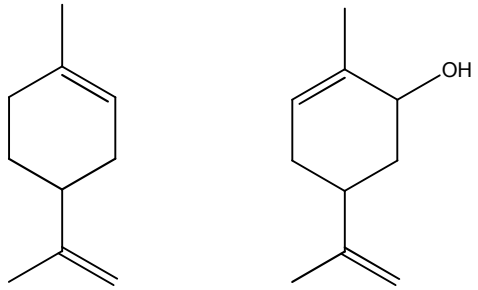

Figure 3: Structures of limonene (left) and carveol (right).

Cloning

DNA from *Rhodococcus* sp. RHA1 was isolated by A. Riebel, and made available for use in the PCR reactions. Isolation of the genomic DNA was based on the large size of the genome (9.702.737 bp), and no confirmation was present whether the three plasmids of *Rhodococcus* sp. RHA1 had been co-isolated. The genes were located on the genome (2x), pRHL1 and pRHL3, making co-isolation of these two plasmids necessary for successful PCR reactions. To omit the problem of co-isolation of the plasmids, the PCR reactions for *mox1* were performed before an attempt was made for the other three genes, due to the location of *mox1* on the genome.

The first PCR reaction (annealing 30 sec. at 55˚C) resulted in fragments of small sizes on gel, indicating insufficient time for the primers to anneal to the template DNA in the correct way. To allow more time during the annealing step, a touch-down cycle (annealing 30 sec. at 62˚C -> 52˚C) was used, resulting in bands on gel of expected size. The other three genes were amplified with the use of the same PCR program as was used for *mox1*, and all three showed bands on gel of expected size. The appearance of bands for *mox2* and *mox3* indicated the co-isolation of plasmids pRHL1 and pRHL3 during DNA isolation along with the genomic DNA.

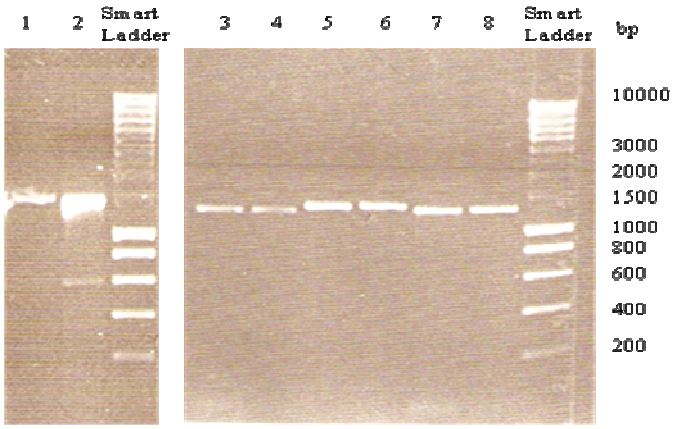

Figure 4: Gel of PCR fragments obtained with the touch-down annealing program. The genes of mox1 (lanes 1 and 2), mox2 (lanes 3 and 4), mox3 (lanes 5 and 6) and mox4 (lanes 7 and 8) are of expected fragment sizes between 1200 and 1500 bp.

The plasmid map of the original pBAD vector is depicted in figure 5, showing the ampicillin resistance gene and the araBad promoter. A pBAD vector containing a PAMO gene in the multiple cloning site (MCS) was used to express all four genes after removing the PAMO gene with restriction enzymes. The advantage of using this plasmid was the modification made to contain a NdeI site. NdeI and HindIII were used to digest the vector for cloning of the genes, thereby removing the PAMO gene and introducing a stopcodon in front the MYC/HIS tag. This resulted in gene expression in its native form and minimizes the occurrence of incorrect folding of the proteins. The pCRE3C vector, derived from pBADNK vector, was also used to N-terminally fuse *mox1* to a cofactor regeneration gene (PTDH). Incorporation of *mox1* in the vector gave a fused protein with a polyhistidine tag for purification.

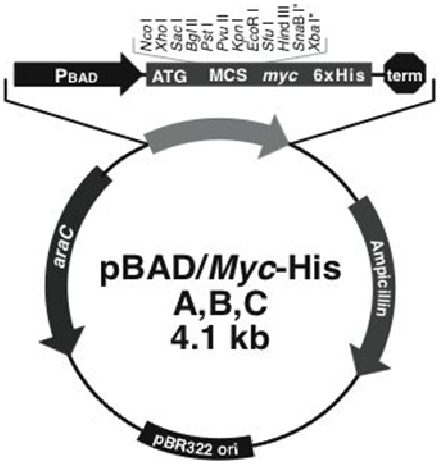

Figure 5: Map of pBADNK indicating all features of the plasmid. The locations are: *ara*BAD promoter region 4-276, initiation ATG 319-321, multiple cloning site 317-370, *myc* epitope 377-406, polyhistidine tag 422-439, *rrn*B transcription termination region 545-702, ampicillin ORF 981-1841, pBR322 origin 1986-2659, and araC ORF 4068-3190. (Invitrogen)

All four genes were cloned in pBAD vectors, and *mox1* was additionally cloned in the pCRE3C vector for possible co-factor regeneration, with the use of ligation independent In-Phusion cloning method. Restricted plasmids and PCR product were incubated with In-Phusion enzyme and subsequently transformed *E. coli* TOP10 cells with the vector. Initial confirmation of successful incorporation of the PCR fragments in the vectors was obtained by restriction analysis on isolated plasmids, showing the expected fragment sizes after digestion. Sequence analysis confirmed successful incorporation of the genes in the pBAD and pCRE3C vectors, and showed the absence of mutations in the sequence of the genes.

Expression and purification

The genes can be expressed by activating the araBad promoter, inducible by the addition of L-arabinose to the growth medium. To optimize the L-arabinose induced overexpression of enzymes, varying concentrations of L-arabinose and temperatures were tested.

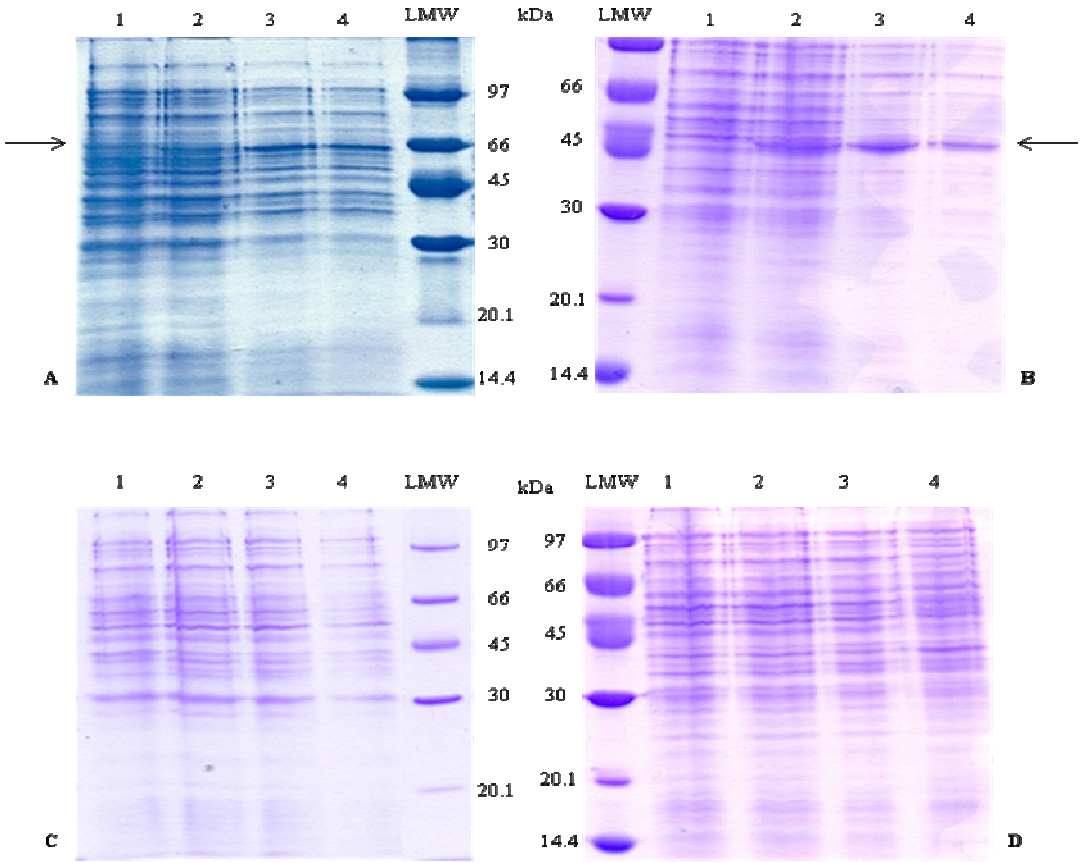

Figure 6: SDS-PAGE gels of cell extract samples obtained during expression optimization tests of the pBAD constructs. Arrows indicate the positions of over expressed protein in the samples. The concentrations of L-arabinose are 0% (lane 1), 0.0002% (lane 2), 0.02% (lane3), and 2% (lane 4). (**A**) Expression of Mox1 with at 24˚C. (**B**) Expression of Mox2 at 30˚C. (**C**) Expression of Mox3 at 30˚C. (**D**) Expression of Mox4 at 30˚C.

Proteins Mox1 (similar expression observed for pCRE3C construct) and Mox2 showed good expression observed in cell extract (CE) samples. However, proteins Mox3 and Mox4 did not show defined bands, as can be seen in figure 6C and 6D. The latter two may be expressed after induction with L-arabinose, having the level of expression being insufficient for visible bands to be present on gel. The proteins may be active in the cell extract, and can be tested for with a substrate screening assay to test conversion to product. The temperature at which Mox1 was best expressed in both vectors was 17˚C to 24˚C at a L-arabinose concentration of 0.02%. In the case of Mox2, the protein was best expressed at 30˚C and 0.02% L-arabinose concentration.

SDS-PAGE analysis of cell extract and cell free extract showed over expression of Mox1 and Mox2 proteins as seen above. The Mox2 protein is visible in both cell extract and cell free extract samples, and can be considered as soluble (figure 7). Mox1 (both pBAD and pCRE3C version) are not visible in the cell free extract, and could be traced to the insoluble pellet fraction.

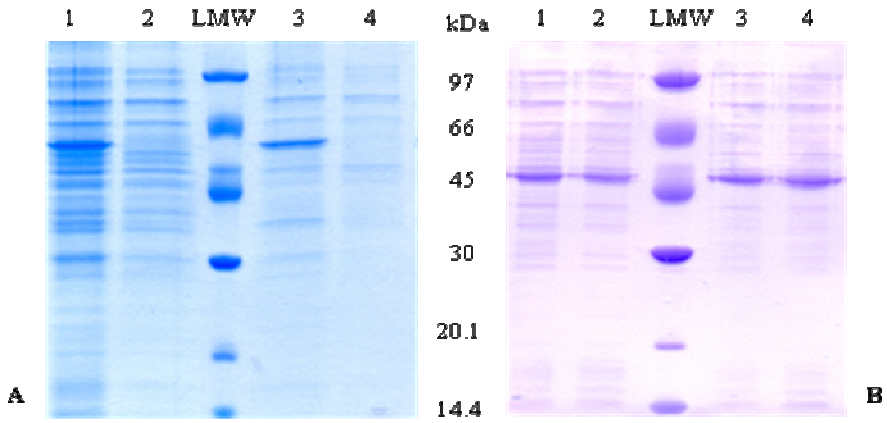

Figure 7: The CE (lanes 1 and 3) and CFE (lanes 2 and 4) samples are shown next to each other for comparison. Expression of protein at 24˚C (lanes 1 and 2) and 30˚C (lanes 3 and 4) were tested to find the best conditions for expression. (**A**) The protein band of Mox1 is clearly visible in the CE lanes and not in the CFE lanes. (**B**) Protein Mox2 is visible in both the CE and CFE samples.

The use of LDAO-buffer, Brij-35, and DMP-buffer instead of 50 mM Tris/HCl pH 7.5 buffer for resuspending the pellet resulted in the same pattern for Mox1 on SDS-PAGE gel, being present in the cell extract lane and not in the cell free extract lane (figure 8A). Protocol used to determine the presence of inclusion bodies confirmed suspicions of inclusion bodies (figure 8B).

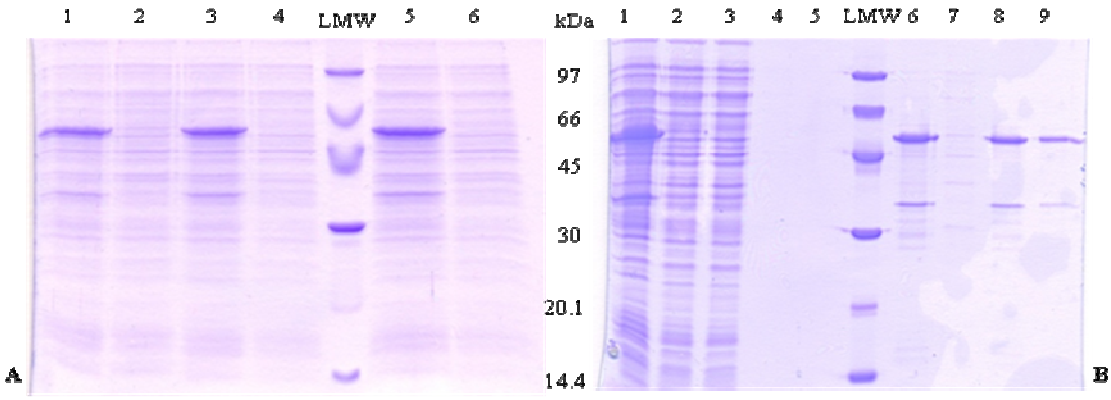

Figure 8: SDS-PAGE gels of solubility tests for Mox1. (**A**) LDAO-buffer (lane 1 and 2), Brij-35 (lane 3 and 4), and DMP-buffer (lane 5 and 6) were used to test the solubility of Mox1 and gave the same result as Tris/HCl buffer. (**B**) Samples taken during the protocol used to determine the presence of inclusion bodies for Mox1. CE sample was taken during expression at 24˚C and 0.02% L-arabinose concentration. CE is loaded in lane 1, soluble fractions in lanes 2 to 5, and pellet samples in lanes 6 to 9 (lane 9 contains inclusion bodies).

Cell free extract, containing over expressed Mox2 protein, was purified with an Äkta – DEAE sepharose column resulting in a purer protein solution coming off at 400 mM KCl. Additional proteins could still be seen on gel, and only a small amount of protein impurities was lost after purification. A slower salt gradient could improve the separation of enzymes over the column to obtain a more pure protein solution. The enzyme solution was desalted and concentrated with an ultrafiltration unit (Amicon) to a volume of 10 ml. Aliquots of 1 ml were stored at -80˚C for further use in substrate screening assays. The concentration of enzyme was not determined before starting the substrate screening tests.

Binding of FMN co-factor to the enzyme was tested by incubating Mox2 solution with FMN for 15 minutes and adding the solution to a separating desalting column. All yellow color went through the column and no color was observed staying on the column and coming off at the same time as the protein. This indicated the protein came off separate from the FMN co-factor fraction. The protein did not bind oxidized FMN cofactor without substrate being present.

Substrate screening

In order to explore the substrate specificity of Mox2, a wide range of potential substrates was selected for investigation, shown in figure 9.

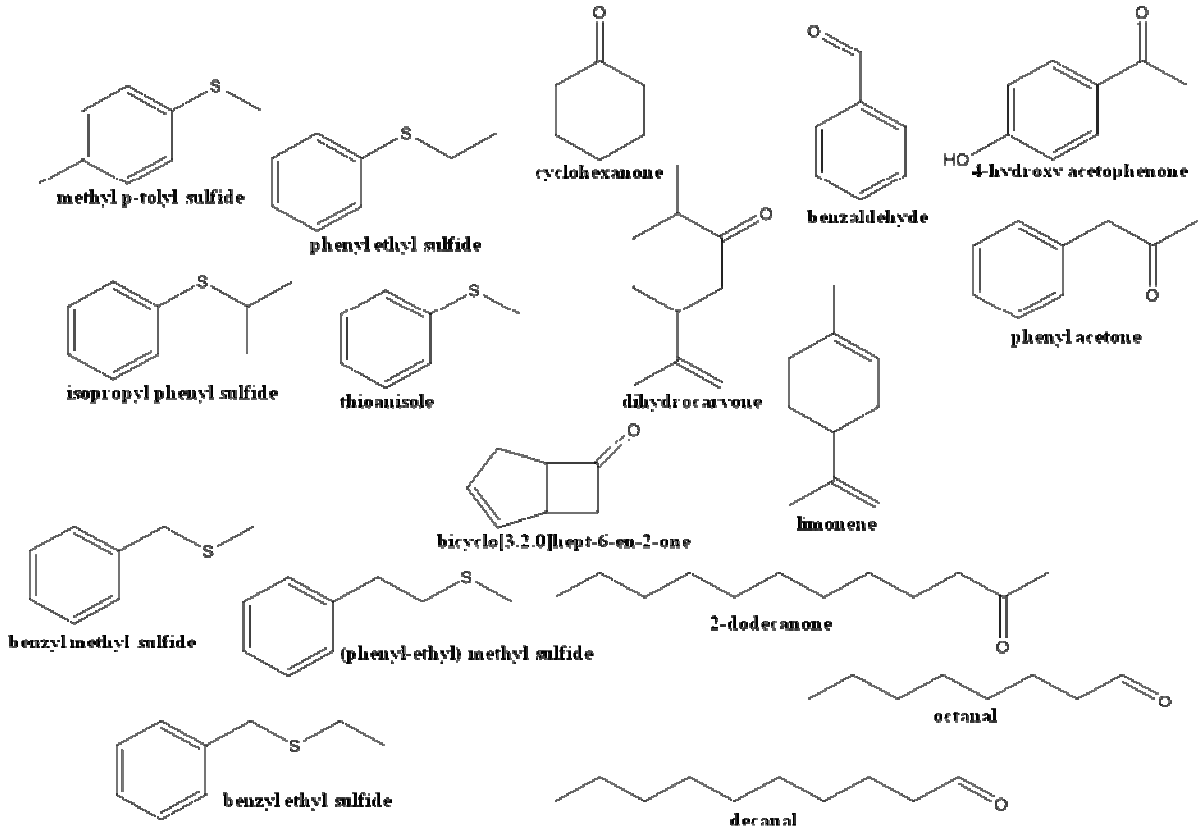

Figure 9: Limonene, sulfides, ketones and aldehydes used for substrate screening.

The previous FMN binding test did not give conclusive result on the preferred cofactor of Mox2. To see whether Mox2 uses FMN or FAD as cofactor UV absorption spectra were recorded at 440 nm wavelength to see if addition of potential substrate had an effect on the absorption. In the case of FMN several compounds had a stabilizing effect on reduced FMN (see appendix C). In the case of FAD no significant change in absorption spectra was observed, and let to the assumption Mox2 was FMN dependent.

The substrate screening assay was performed for several ketones, aldehydes and limonene listed in table 3. The standard reaction conditions, as described above, resulted in the identification of two compounds giving conversion to a product. For Bicyclo[3.2.0]hept-6-en-2-one the corresponding lactones were observed after the reaction time was increased to 62 hours, but GC-MS measurements for dihydro-carvone did not confirm the formation of lactones. For octanal and decanal the formation of the corresponding hydroxides was observed and not expected to be a product of a BVMO.

Table 3: Conversions of limonene, ketones and aldehydes by Mox2.

Substrate	**Time (hours)**	**Conv. (%)**	***ee* (%)**	**Ratio lactones***
Bicyclo[3.2.0]hept-6-en-2-one	62	17	n.d	84:16
Dihydro-carvone	62	8**	n.d	n.d
Octanal	3	>99**		
Decanal	3	>99**		
Limonene	62	-		
2-dodecanone	62	-		
Cyclohexanone	62	-		
Phenyl acetone	4	-		
4-hydroxyacetophenone	4	-		
Benzaldehyde	4	-		

* Ratio between "normal/abnormal" lactones.
** Observed conversions were different products than expected for Baeyer-Villiger reactions.

The conversion of ketones and aldehydes, as well as the substrate of the original protein (limonene), showed almost no conversion upon addition of enzyme. Bicyclo[3.2.0]hept-6-en-2-one showed the best result with a 17% conversion after 62 hours of incubation. Bicyclo[3.2.0]hept-6-en-2-one is known for its acceptance by many BVMOs, and led to believe Mox2 had monooxygenase activity.

In addition to oxidation of ketones and aldehydes, some BVMOs are also able to catalyze the sulfoxidation of a variety of sulfides, which can be interesting chiral building blocks for pharmaceutical chemistry [Pazmiño, 2008]. The seven sulfides tested are listed in table 4, showing conversion to the S-enantiomer for five sulfides with high enantioselectivity. For three of the substrates a reaction time of two hours was sufficient to observe product formation. The remaining for substrates were tested for conversion by increasing the reaction time. After 6 hour reactions there was still no product formation detected and the reaction time was raised to 24 hours. In two cases, the increased reaction time to 24 hours resulted in the observed product formation.

Table 4: Conversion of sulfides by Mox2.

Substrate	**Time (hours)**	**Conv. (%)**	***ee* (%)**	**Configuration***
Thioanisole	2	4	85	S
Ethyl Phenyl Sulfide	2	9	71	S
Benzyl Methyl Sulfide	2	6	94	S
(Phenyl- Ethyl) Methyl Sulfide	24	-		
Benzyl Ethyl Sulfide	24	4	84	S
Isopropyl Phenyl Sulfide	24	-		
Methyl p-Tolyl Sulfide	24	19	94	S

* Based on the conversion by HAPMO.

In order to optimize the reaction conditions of the assay and increase the conversions by Mox2 enzyme, several adaptations compared to the standard protocol were tested for conversion of thioanisole, see table 5.

Table 5: Variations on reaction conditions for conversion of substrate thioanisole by Mox2. The concentration of NADH (6 mM) and thioanisole (1 mM) were kept constant.

Difference	**Reaction mixture***						**Time in hours**	**Conversion in %**
	FMN Concentration	*Glucose-6-Phosphate*	*Catalase*	*Reductase*	*Temperature in ˚C*	pH		
Concentration of FMN co-factor	0 µM	-	-	-	17	7.5	2	0
	0.25 µM	-	-	-	,,	,,	2	0
	2.5 µM	-	-	-	,,	,,	2	0
	25 µM	-	-	-	17	,,	2	2
	250 µM	-	-	-	17	,,	2	4
Reductase	25 µM	-	-	0		7.5	1	1
	,,	-	-	1 µl		,,	1	2
Glucose-6-Phosphate with G-6-P Dehydrogenase (1 unit) and catalase	25 µM	0 mM	-	-	17	7.5	24	12
	,,	5 mM	-	-	,,	,,	24	22
	,,	-	0	-	,,	,,	6	0
	,,	-	1 unit	-	,,	,,	6	17
	,,	0 mM	0	-	,,	,,	6	7
	,,	5 mM	1 unit	-	,,	,,	6	7
Concentration of FMN and temperature	25 µM	-	-	-	17	7.5	2	1
	25 µM	-	-	-	30	,,	2	1
	25 µM	-	-	-	37	,,	2	2
	250 µM	-	-	-	17	,,	2	4
	250 µM	-	-	-	30	,,	2	2
	250 µM	-	-	-	37	,,	2	4
pH	25 µM	-	-	-	17	7	2	3
	,,	-	-	-	,,	7.5	2	3
	,,	-	-	-	,,	8	2	2
	,,	-	-	-	,,	8.5	2	2
	,,	-	-	-	,,	9	2	2

* All reaction mixtures contained the same amount of enzyme solution.

The variation of reaction conditions let to an improvement compared to the standard protocol when changing temperature and FMN concentration. The test further confirmed the suspicion that FAD was not a co-factor for Mox2, as it showed no conversion in the GC measurements. The pH optimum 7.5 in Tris/HCl buffer was considered to be the best choice, because the conversion was slightly better compared to the reaction at pH 7.0 and pH 8.0. Conversions without FMN, NADH or Mox2 added gave no conversions, eliminating the possibility of auto conversion or conversion by other enzymes in the Mox2 solution.

To see if the high conversions observed for octanal and decanal were due to Mox2 luciferase activity, the reactions were repeated in a dark room for observation of produced light. The reaction with decanal/octanal showed no light emission. The conversion of both molecules has to be further investigated to make sure the conversion can be attributed to Mox2.

Discussion

The metabolic diversity of *Rhodococcus* sp. RHA1 has been reported by McLeod et al. to have a very wide range, containing three additional plasmids along the genome with mainly metabolic genes. In silico screening of the genome of *Rhodococcus* jostii RHA1 with a known BVMO motif resulted in at least 20 putative Type I BVMO genes, which are being cloned and screened for activity. The large sequence length of the *Rhodococcus* sp. RHA1 genome suggests more putative BVMO genes e.g. also Type II might be present, and might need a different approach to be detected by screening. Protein BLAST of the *Rhodococcus* sp. RHA1 genome with the sequence of LadA and LimB resulted in four genes with good sequence similarity (>40%). For further characterization, all four genes were successfully cloned in expression vectors with an araBad promoter.

Expression induced by addition of L-arabinose, resulted in observable overexpression of two proteins, and the other two proteins could not be identified on gel (shown in figure 4). The latter two proteins can either have the problem of being expressed in very low quantities, thereby making it difficult to observe them on gel, or are not expressed at all or degraded. A change of expression vector, with a differently induced promoter (e.g. IPTG), might be sufficient to observe overexpression compared to the proteins of *E. coli*. A second possibility, is to test cell extract on possible BVMO activity by testing possible substrates, which have been accepted by a large range of BVMOs (e.g. bicyclo[3.2.0]hept-6-en-2-one or indole). Codon optimization or changing the host for expression could also be tried to get overexpression of the proteins.

Although Mox1 could be overexpressed in *E. coli* cells, the protein failed to stay soluble. The sequence gave no indication of the protein being associated to the membrane of the cells, which would pull the protein into the insoluble fraction after centrifugation. Inclusion body formation was a more logical option, and could be determined by a simple test. To overcome the problem of inclusion body formation the use of chaperone proteins being expressed together with Mox1 might help in correct folding of Mox1. Fusion with a stabilizing protein has to be carefully examined, because CRE3C fusion had no visible effect on solubility. Lower temperature for expression of Mox1 will be difficult to achieve due to the already low temperature of 17˚C during the expression tests.

Mox2 was overexpressed in *E. coli* cells and remained in solution after centrifugation of the cell extract. Purification of Mox2 over the Äkta – DEAE sepharose column resulted in elution of the protein at 400 mM KCl in a broad peak (Appendix B). The protein peak showed bumps and other small peaks visibly overlapping, indicating more proteins could have come off at the same moment, and was supported by residual reductase activity. For better controlled substrate screening assays a better purified enzyme would be desirable to eliminate uncontrolled activity. The concentration of reductase is unknown, and pure Mox2 solution would allow for fixed amount of reductase to be added.

Mox2 was found to catalyze the oxidation of sulfur containing substrates with good enantioselectivity, resulting in the formation of mainly the (S)-sulfoxides. The configuration was based on comparison with conversions performed by available HAPMO enzyme of the same substrates, the data being available in literature [Pazmiño, 2008]. The best improvement of conversion rate of thioanisole was achieved by increasing the duration of the reaction. All other tested conditions for conversion had no or small impact on total conversion of the substrate.

The remaining compounds tested for conversion by Mox2 showed product formation for decanal, octanal, bicyclo[3.2.0]hept-6-en-2-one, and dihydro-carvone. GC-MS analysis of the products formed for decanal and octanal showed products possibly formed by residual proteins of *E. coli*, and not monooxygenase activity. For bicyclo[3.2.0]hept-6-en-2-one and dihydro-carvone long conversion times were needed to observe a low conversion to product. Conversion tests without NADH or FMN showed no conversions, indicating no auto oxidation had occurred. To be able to tell whether both compounds are real substrates for Mox2, better reaction conditions have to be determined.

Further characterization of the substrate specificity of Mox2 is needed to determine the function of the enzyme, and the preferred substrate for conversion. Improved reaction conditions should yield higher conversions by Mox2, and allow the determination of catalytic parameters (k_{cat}, K_m and k_{cat}/K_m) to show how good the enzyme functions. Thermostability and monomer/dimer/tetramer as active formation in solution can be tested, for even better characterization of the enzyme.

Conclusion

The increasing amount of deposited genome sequences in online databases facilitates screening for interesting genes. In order to broaden the range of recognized substrates by available BVMOs, genome mining of *Rhodococcus* sp. RHA1 with the sequence of a known Baeyer-Villiger monooxygenase and n-alkane degrading monooxygenase was performed. Three putative Type II BVMO genes and one putative n-alkane degrading gene with good sequence similarity were identified on the genome and accompanying plasmids. Cloning and expression of the genes in *E. coli* resulted in the purification of one soluble protein, Mox2.

Substrate screening assays revealed enantioselective conversion of sulfides (S-configuration) and bicyclo[3.2.0]hept-6-en-2-one (normal lactone). The amount of conversion and substrate range have to be tested at a fixed protein concentration. The observed conversions can be better compared and differences between several conditions better explained by knowing the concentration. Further attempts will be made to (over)express and purify the remaining three proteins, thereby increasing the effectiveness of the in silico screening approach. In addition, the in silico screening of the genome of *Rhodococcus* jostii RHA1 reduces the time of work compared to isolating and cultivating organisms or constructing metagenome libraries.

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Appendices

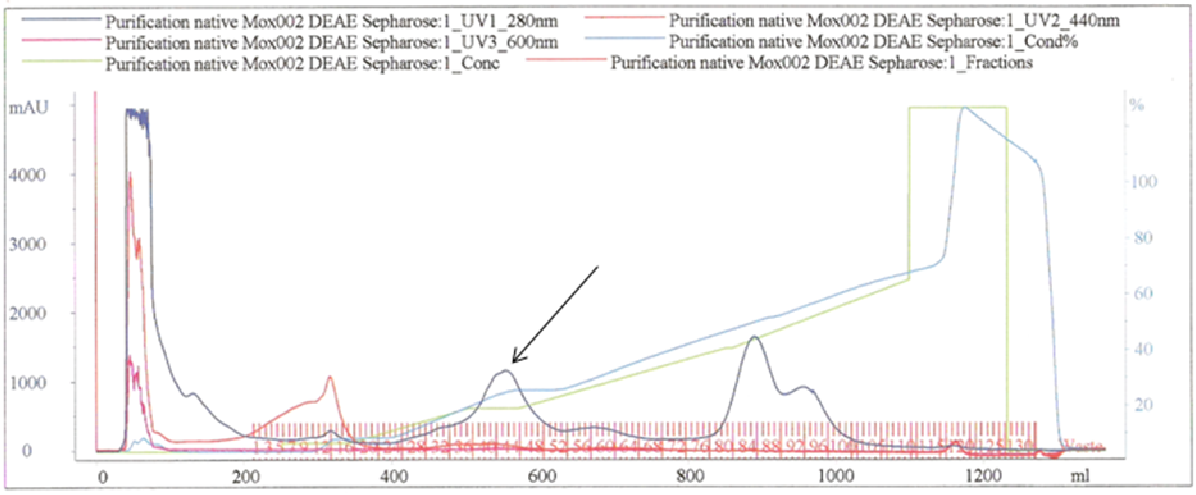
Appendix A_1: Sequence alignment of LadA and Mox1.

```
LadA            MTKKIHINAFEMNCVGHIAHGLWRHPENQRHRYTDLNYWTELAQLLEKGKFDALFLADVV 60
Mox1            MTRQIRFNAFDMNCVAHQSPGLWRHPDDQSHRYTDLGYWTDLARLLERGLFDGLFIADVL 60
                **::*::***:****.* : ******::* ******.***:**:***:* **.**:***:

LadA            GIYDVYRQSRDTAVREAVQIPVNDPLMLISAMAYVTKHLAFAVTFSTTYEHPYGHARRMS 120
Mox1            GTYDVYGGTDEAALRQGAQIPVADPLLLVSAMAAVTEHLGFGITTGTGFEHPYPFARRLS 120
                * ****  : ::*:*:..**** ***:*:**** **:**.*.:* .* :**** .***:*

LadA            TLDHLTKGRIAWNVVTSHLPSADKNFGIKKILEHDERYDLADEYLEVCYKLWEGSWEDNA 180
Mox1            TLDHLTGGRIGWNVVTGYLPSAARNFGDADQLDHDTRYDHADEYLEVLYKLWEGSWEDDA 180
                ****** ***.*****.:**** :***  . *:** *** ******* **********:*

LadA            VIRDIENNIYTDPSKVHEINHSGKYFEVPGPHLCEPSPQRTPVIYQAGMSERGREFAAKH 240
Mox1            VVRDTERGVYVDPEKVHHIGHRGTHFTVPGIHLSEPSPQRSPVIYQAGASPRGVRFAAEN 240
                *:** *..:*.**.***.*.* *.:* *** **.******:******* * ** .***::

LadA            AECVFLGGKDVETLKFFVDDIRKRAKKYGRNPDHIKMFAGICVIVGKTHDEAMEKLNSFQ 300
Mox1            AEAIFVGPPSKRVLKDTVARIRQALVDAGRDPYSARIYALSTVITGSTDEAAAAKQEEYR 300
                **.:*:*  . ..**  *  **:   . **:*   :::*   **.*.*.: *  * :.::

LadA            KYWSLEGHLAHYGGGTGYDLSKYSSNDYIGSISVGEIINNMSKLDG-------------- 346
Mox1            RYADLEGALVFTSGWMGIDLSRYDLDDPIGNVESNAIQSAVAAFQEASDDGREWTVRDIA 360
                :* .*** *.. .*  * ***:*. :* **.:. . * . :: ::

LadA            KWFKLS------VGTPKKVADEMQYLVEEAGIDGFNLVQYVSPGTFVDFIELVVPELQKR 400
Mox1            EWASIGGLGPRFVGSGETVATQLQEWVADTDVDGFNLAYAITPGSFEDVVTHVVPALQAR 420
                :* .:.      **: :.** ::*  * ::.:*****.  ::**:* *.:  *** ** *

LadA            GLYRVDYEEGTYREKLFGKGNYRLPDDHIAARYRN---ISSNV--------- 440
Mox1            GAYPTGYTDGTLRHKLFDKGD-RLPADHRGARYRLGGDLSTRTDRYRAYTNN 471
                * * ..* :** *.***.**: *** ** .****    :*:..
```

Appendix A_2: Sequence alignment of LimB, Mox2, Mox3 and Mox4.

```
Lim.        ----------------------------MTDDFGRVDFGAFLAPWHR-ADSDANFAIHQD 31
Mox2        ----------------------------MSESNYPTAFGVFMAPFHK-IGVSPTVLYERD 31
Mox3        MFSCANIPYEALQSSADPASKTPASRTPARELNMALKFGAFMAPFHCPVGQDPTYAIERD 60
Mox4        ------------MEPEAGNNGSSVNNAPTSRKAPLRCGVFLPPYHN-PRRNPTMAIEQD 46
                                      .      *.*:.*:*     ...   .:*

Lim.        LELVEHLDRLGFAEFWLGEHHSGGVEIVASPEMFMAAAAQRTQRIKLGLGVVSLPYHHPF 91
Mox2        LQIIELADSLGFQEAWLGEHHSGGHEPIGAPEMLLAAAAQRTKTIKLGTAVNSLPYHNPF 91
Mox3        LSVLRHLDMLGFEEAWIGEHHSCGTELVPDPFIFIAYAAQETRHIKLGTGVVSLPYHNPL 120
Mox4        LRHVEDLDRLGFHQVWFGEHHSGGYEVGPSPELMIAAAAQRTSRIELCSGVISLPYHHPL 106
            *  :.  * *** : *:***** * *    * :::* ***.*  *:*  .* *****:*:

Lim.        LVADRLVLLDHLSRGRMIFGAGPGQLADDAKMLGIDPIDSRRKMEEAFDVIHRLLAGE-T 150
Mox2        LLAERLVTLDHLSRGRAIMGFGPGQLLSDAYMLGIDPTKQRDMMLEAAEVIVRLLRGE-T 150
Mox3        WIADRALFTDRLLRGRFMLGLGPGSLPTDAAMIGISMAEQRGAFEEDVDVLMAILRGE-S 179
Mox4        MVADRITFLDHMTRGRIRIGVGPGALVADSQMMGMDYNLLRPRMEESLDAILELLNTPGP 166
             :*:*    *:: ***  :* *** *  *: *:*:.    *  : *  :.:  :*    .

Lim.        VTQKTDWFTCQDAYLHVAPYSN--IQKAVTATVSPTGPKLAGKYGSGILSLAATNPVGVE 208
Mox2        VTEKTDWFDLREAKLQVRPYSLPELDVVVAAVTSPSGPVTAGRLGVGMINLAATSPAAFG 210
Mox3        VTHETPRYRLHDARTQFAPYSD--FEITVAAVATPTGPRVAAKNGLNLMSVGATAQGGFD 237
Mox4        VDRKTEWFSLEDARVQLGPYSYPGVPITVAAAMSPSGPKLAGKYGLGLLSIGGTSAKSVE 226
            * .:*  :  .:*  :. ***   .  .*:*. :*:**  *.: * .::.:..*   ..

Lim.        KLAEHWKIAEDIAAENGQTVDRADWRLSGIMHVAETEEQARADVRHGLLYLMNYLSNITP 268
Mox2        ALRGHWKIAEDEAAKAGRTVSRRNWRLSAITHIAETEEQARKDCTHGFEEIWNYLAHISP 270
Mox3        ALALHWDVMEERAPQFGHTPDRAGWRLVGPMHLAETKEQAIEDVRHGLDAWCHYSQHILA 297
Mox4        LLAQTWDITTHEANVYGQSVDRNDWAIVGNIHIADTFEQAVEDTRFGLRDFMDYRHVVTP 286
             *   *.:  . *   *:: .* .* : .  *:*:* ***  *  .*:    .*   : .

Lim.        G-FAAAP---DVDSLIDGINDAGLAVIGTPEMAVTQIRRLQEKSGGFGKFLVLHGEWAST 324
Mox2        LPASEKP---DVEGMIDEAIESGSVLIGTPDTAIKKIRQLADQTGGFGSFIMTLTDFTDY 327
Mox3        APHFRAVG-ETFEERVDWINETGLGVIGTPDDAVRQIERLEEQSKGFGSYLLLHHEWARH 356
Mox4        MKMIEPGEEVAHEELVRRVNESGYGCIGDANDAIDYIQKIIDRTGGFGTFLATAHDWADI 346
                       :  :    ::*   ** .: *:  *.:: ::: ***.::    :::

Lim.        TAALHSFELIAQQVAPHFNGDLGPRLRGYN-QTMNSNRSAADITQAAQEEAQKRFEAERA 383
Mox2        ERQRNAVTLWAEHVIPEFRGQLKP-IREHN-AWVRGNEWKAETMAAINAASEKYAAQQQP 385
Mox3        DAVLKSYELFANHVKPRFQGSTSRLQKAEDYAVSRWAELDKRQGDAIQAATDRHVKERAA 416
Mox4        EATSRMYRILMRDVMHRFTGTCEPLLNSYHWTQARKEGFSERFATGISQATQDYERAKSA 406
                .   :  ..*  .* *      .  .    .          . .  ::     . .

Lim.        IRTN 387
Mox2        VSVN 389
Mox3        AGRA 420
Mox4        G--- 407
```

Appendix B: Graph recorded during purification of Mox2 (arrow indicates protein peak).



Appendix C: UV spectroscopy measurement of reduced FMN stability in the presence of possible substrates at 440 nm.