

# Cloning, expression and characterization of a Baeyer-Villiger monoxygenase from *Pseudomonas putida* KT2440

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**Abstract** The gene encoding a Baeyer-Villiger monoxygenase and identified in *Pseudomonas putida* KT2440 was cloned and functionally expressed in *Escherichia coli*. The highest yield of soluble protein could be achieved by co-expression of molecular chaperones. In order to determine the substrate specificity, biocatalyses were performed using crude cell extract, growing and resting cells. Examination of aromatic, cyclic and aliphatic ketones revealed a high specificity towards short-chain aliphatic ketones. Interestingly, some open-chain ketones were converted to the alkylacetates, while for others formation of the ester products with oxygen on the other side of the keto group could also be detected yielding the corresponding methyl or ethyl esters.

**Keywords** Aliphatic ketones · Baeyer-Villiger monoxygenase · Cloning · Enzymatic reactions · *Pseudomonas putida*

## Introduction

Baeyer-Villiger monoxygenases (BVMOs) belong to the class of oxidoreductases and convert aliphatic, cyclic and aromatic ketones into esters and lactones, respectively, using molecular oxygen (Mihovilovic et al. 2002). They are flavin-dependent (mostly FAD) and require NAD(P)H to catalyze this reaction. The cyclohexanone monoxygenase from *Acinetobacter calcoaceticus* NCIMB 9871, converting mono- and bicyclic ketones (Donoghue et al. 1976, Stewart 1998), is the best studied BVMO so far. Other BVMOs converting aromatic ketones (Kamerbeek et al. 2001, Tanner and Hopper 2000) or aliphatic open-chain ketones (Britton and Markovetz 1977, Malito et al. 2004) were also described. Recently, the gene of a BVMO from *Pseudomonas fluorescens* DSM 50106 was cloned and functionally expressed in *E. coli* (Kirschner et al. 2007). This enzyme preferentially accepts aliphatic acyclic 2-ketones and converts racemic 4-hydroxy-2-ketones with good enantioselectivities (Kirschner and Bornscheuer 2006). In 2004 the first crystal structure of a BVMO from *Thermobifida fusca*, the phenylacetone monoxygenase, was published (Malito et al. 2004).

In 2002 the complete genome sequence of *Pseudomonas putida* KT2440 was published (GenBank accession number: AE015451) (Nelson et al. 2002). 15 open reading frames encoding putative monoxygenases were determined. Sequence analysis on protein and nucleotide level revealed one monoxygenase

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containing sequence motifs typical for Baeyer-Villiger monooxygenases (consensus sequence (FXGXX XHXXXW) and binding sites for cofactor and prosthetic group (GXGXXG-motifs)) (Fraaije et al. 2002). In this paper, we now describe the cloning, expression and the biocatalytical investigation of this newly found Baeyer-Villiger monooxygenase.

## Materials and methods

### Bacterial strains and plasmids

*E. coli* DH5 $\alpha$  was from Clontech, *E. coli* JM109 was received from New England Biolabs, *E. coli* BL21 (DE3) and pET22b(+) were purchased from Novagen. *Pseudomonas putida* KT2440 was obtained from the FZ Jülich (Jülich, Germany). The Chaperone Plasmid Set containing the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16 was purchased from TaKaRa Bio Inc.

### Construction of expression vectors

The BVMO gene was amplified from the genomic DNA from *Ps. putida* KT2440 without the stop codon using the polymerase chain reaction (PCR) with the oligonucleotides FW-*Pp*KT2440-gDNA (5'-GTA CAT CGA TGG GAT CCT CGG CGG-3') and RV-*Pp*KT2440-gDNA (5'-GTA CGA GCT CCC ATA TGT CCT CTC ACA C-3') and *Pwo* DNA polymerase (Roche). The restriction sites for *Nde*I and *Bam*HI (underlined) in the primer sequences were used to perform a directed cloning between the *Nde*I/*Bam*HI sites of the vector pJOE4072.6 (Kirschner et al. 2007). Hereby the BVMO gene was fused at the C-terminal end in frame to the six histidine codons (coding for a His-tag) following the *Bam*HI site in the vector. This plasmid was named pJOE-KT2440. For cloning into pET22b(+), a *Hind*III restriction site within the gene was deleted using QuikChange site-directed mutagenesis (Stratagene). The mutated pJOE-KT2440 was then digested with *Nde*I and *Hind*III and the resulting fragment, consisting of the BVMO gene and the His-tag, was cloned into pET22b(+). This construct was named pET22b(+)-KT2440. Constructs without C-terminal His-tags were obtained by introducing a TGA

stop-codon into the *Bam*HI site using QuikChange site-directed mutagenesis.

### Gene expression

*E. coli* JM109 was transformed with the pJOE construct, BL21 (DE3) with the pET22b(+) construct. In both cases expression was performed in 30 ml LB media containing 100  $\mu$ g ampicillin/ml (LB<sub>amp</sub>) at 25, 30 and 37°C. At an optical density (600 nm) of 0.6–0.7, BVMO expression was induced by the addition of L-rhamnose (0.2% w/v final concentration) for the pJOE construct and with 0.5 mM IPTG for the pET22b(+) construct. During growth for up to 24 h, samples were taken, centrifuged, resuspended in 200  $\mu$ l sodium phosphate buffer (50 mM, pH 7.5) and disrupted on ice by sonication (Sonoplus, Bandelin, Germany; 50% pulse and 50% amplitude). Inclusion bodies were separated from the cell lysate by centrifugation for 10 min (4°C, 800  $\times$  g), treated with 0.2% (v/v) Triton X-100 (10 min, 37°C) and washed twice with sodium phosphate buffer. Cell debris were removed from the cell lysate by additional centrifugation at high speed (10 min, 4°C, 16,000  $\times$  g). Soluble and inclusion body fractions were analysed by SDS-PAGE and Western blotting.

### Coexpression of the BVMO gene with chaperone encoding plasmids

*E. coli* JM109 and BL21 (DE3) cells were transformed with chaperone encoding plasmids and competent cells were prepared thereof. These were transformed with pJOE constructs (for JM109) or pET22b(+) constructs (for BL21 (DE3)) and selected on LB-plates containing 100  $\mu$ g ampicillin/ml and 34  $\mu$ g chloramphenicol/ml (LB<sub>amp + cm</sub>). Expression was performed at 30°C as described before using LB<sub>amp + cm</sub> containing 0.5 mg L-arabinose/ml (in case of pGro7, pKJE7 and pTf16), 5 ng tetracycline/ml (in case of pG-Tf2) or L-arabinose and tetracycline (in case of pG-KJE8) in concentrations given above.

### Biocatalysis and biotransformation

Substrate specificity was investigated using crude cell extract, growing and resting cells. For all experiments *E. coli* JM109 pJOE-KT2440 pGro7 cells were used.

### *Biocatalysis with crude cell extract*

Cells were grown in 2x YT<sub>amp + cm</sub> medium at 37°C to an optical density (600 nm) of 0.6–0.7. BVMO expression was induced by the addition of L-rhamnose to 0.2% (w/v), chaperone expression (GroES/EL) by the addition of L-arabinose to 0.5 mg/ml. Expression was performed at 25°C overnight (~12 h). Cells were harvested at an optical density (600 nm) of 6–7, washed and resuspended in sterile sodium phosphate buffer (50 mM, pH 7.5). Cell disruption was performed by sonication on ice, cell debris were removed by centrifugation (30 min, 4°C, 10,000 × g). Biocatalysis reactions were performed in 2 ml reaction tubes closed with air permeable caps (Lid<sub>Bac</sub>, Eppendorf) at 20, 25 and 30°C. For all investigated temperatures the same crude cell extract was used. To 900 µl crude cell lysate 50 µl FAD (1 mg/ml), 200 mM NADPH and 50 mM substrate (aliphatic, aromatic and cyclic ketones) were added. After defined time intervals (1, 2, 4 and 6 h) samples were taken, extracted with dichloromethane and dried over anhydrous sodium sulphate. Samples were analysed via GC-MS.

### *Biotransformation with resting cells*

Cultivation and BVMO expression were performed as described for crude cell extract. At an optical density (600 nm) of 6–7, cells were harvested, washed twice with sterile sodium phosphate buffer (50 mM, pH 7.5) and resuspended in the same buffer. 10 mM substrate and 20 mM glucose for cofactor regeneration were added simultaneously. Biotransformation was performed in flasks (50 ml cell suspension; optical density 6–7) at 20, 25 and 30°C. Samples were taken at defined times (0.5, 1, 2, 4, 8, 24 and 32 h) and treated as above.

### *Biotransformation with growing cells*

Cells were grown in 50 ml 2x YT<sub>amp + cm</sub> at 37°C to an optical density at 600 nm of 0.6–0.7. BVMO expression was induced by addition of L-rhamnose to 0.2% (w/v) and chaperone expression (GroES/EL) by the addition of L-arabinose to 0.5 mg/ml. Subsequently, 10 mM substrate was added. Expression and biotransformation were performed at 20, 25 and

30°C. After defined time intervals (1, 4, 8 and 24 h) samples were taken and treated as described above.

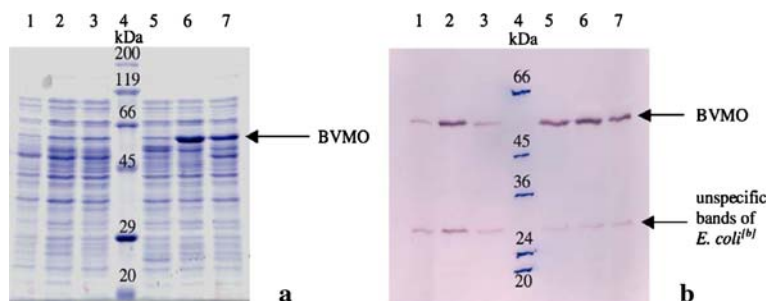
### GC-MS analysis

GC-MS analyses were carried out on a GCMS-QP 2010 instrument (Shimadzu) with a BPX5 column (Macherey-Nagel). All substrates, methyl and ethyl esters were purchased from Fluka at GC standard purity. Other ester standards for GC-MS analysis were prepared enzymatically according to Kirschner et al. (2007). <sup>1</sup>H-NMR spectra were recorded in CDCl<sub>3</sub> on a 300 MHz (Bruker) instrument. The obtained NMR spectra of aliphatic esters matched literature data.

## Results and discussion

### Cloning and BVMO expression in *E. coli*

The gene of the BVMO from *Pseudomonas putida* KT2440 was successfully amplified by PCR from the genomic DNA and inserted into pJOE4072.6 and pET22b(+). The BVMO expression was performed in *E. coli* JM109 with pJOE-KT2440 and in BL21 (DE3) with pET22b(+)-KT2440. Different expression temperatures were investigated and the amount of BVMO in the soluble and inclusion body fraction was studied by SDS-PAGE and western blotting. For both systems, more enzyme was found in the pellet than in the soluble fraction revealing a problem with the proper folding of the BVMO in *E. coli*. Therefore, the BVMO was co-expressed at 30°C with different combinations of chaperones provided by the TaKaRa Chaperone Plasmid Set. It has already been reported that coexpression of molecular chaperones in the *E. coli* cytoplasm could significantly increase the yield of soluble protein (Lee et al. 2004). For co-expression in *E. coli* JM109, the GroES/GroEL complex (pGro7) as well as GroES/GroEL together with DnaK/DnaJ/GrpE (pG-KJE8) or the trigger factor (pG-Tf2) led to noticeably higher amounts of soluble BVMO (Fig. 1), whereas the DnaK/DnaJ/GrpE (pKJE7) alone or in combination with the trigger factor (pTf16) showed only a slightly positive effect on BVMO expression (data not shown). For coexpression in BL21 (DE3) no noticeably higher



**Fig. 1** SDS-PAGE and Western blot of the soluble fraction of BVMO expression in *E. coli* JM109 with and without coexpression of chaperones [a]; for detection of 6xHis tagged BVMO, the QIAexpress detection system using a Ni-NTA-alkaline phosphatase conjugate (Qiagen) was applied according to the manufacturer's instructions. *Notes:* [a] Lanes 1–3: expression without chaperones at 25, 30 and 37°C, respectively; lanes 5–7: chaperone coexpression with pG-KJE8,

pGro7 and pG-Tf2 at 25°C, respectively; lane 4: (a) RotiMark Standard (Roth), (b) Low Range Marker (Sigma). [b] Western blot analysis of expression experiments with *E. coli* JM109 pJOE without an additional gene revealed a band of the same molecular weight assuming an *E. coli* host-protein with cross-reaction to the Ni-NTA-alkaline phosphatase conjugate (data not shown)

amount of soluble BVMO could be obtained (data not shown).

#### Substrate specificity

In order to investigate the substrate specificity of the BVMO from *Ps. putida* KT2440 biocatalytic reactions at 30°C using crude cell extract were performed. Different substrates (cyclic, aromatic and aliphatic open-chain ketones) were investigated. However, only aliphatic acyclic ketones were converted to the corresponding esters with good conversions, while oxidation of cyclic ketones (cyclohexanone, cyclopentanone and cycloheptanone) was rather low and aromatic ketones (acetophenone and derivatives) were not accepted at all (data not shown). In fact, a search in the protein sequence data base revealed that one of the closest homologs (52% sequence identity) with known substrate specificity is the prodrug activator EtaA from *Mycobacterium tuberculosis* (Fraaije et al. 2004). This enzyme was shown to convert aliphatic open-chain ketones as well as phenylacetone and benzylacetone, while typical BVMO substrates like acetophenone and cyclohexanone were not accepted. Interestingly, sequence identity with a BVMO from *Ps. fluorescens* DSM50106 with similar substrate specificity was only 25% (Kirschner et al. 2007).

Further experiments investigating the conversion of aliphatic open-chain ketones (C<sub>8</sub>–C<sub>13</sub>) were then performed at 20, 25 and 30°C using crude cell

extract, growing and resting cells. When using whole cells expressing the BVMO, there was no need to add NADPH, which makes biotransformations cost-effective. Reaction courses were monitored via GC-MS analysis.

In Table 1, conversions at 25°C after 8 h reaction time for all investigated aliphatic acyclic ketones using the three different systems are summarized. As a control, biotransformations were also performed under the same reaction conditions using a pJOE-vector without the BVMO gene, but no ester formation could be detected. Additionally, no other by-product could be found and product esters were not further hydrolyzed by *E. coli* enzymes since no alcohol could be detected. However, especially for the shorter chain compounds (C<sub>8</sub>, C<sub>9</sub>) a significant loss of substrate and product due to evaporation was evident at 30°C.

Apparently, in all three systems the BVMO from *Ps. putida* KT2440 preferentially converted short-chain ketones like 2-decanone, 3-decanone and 4-decanone. Here conversions reached the highest values. A BVMO from *Ps. cepacia* also converts aliphatic open-chain ketones with good yields. However, in contrast to the BVMO from *Ps. putida* KT2440, this 2-tridecanone monooxygenase preferred medium-chain ketones from C<sub>12</sub>–C<sub>14</sub> and also converted cyclopentanone with high specific activity (Britton and Markovetz 1977).

Comparing biocatalysis with crude cell extract and biotransformation using whole cells, either growing

**Table 1** Conversion of aliphatic acyclic ketones using crude cell extract, growing and resting cells

Substrate	Conversion [%]			Product composition (for resting cells)
	Cell extract <sup>a</sup>	Growing cells <sup>b</sup>	Resting cells <sup>c</sup>	
2-Octanone	0	0	4	Hexyl acetate
2-Nonanone	13	n.d.	22	Heptyl acetate
2-Decanone	12	36	93	Octyl acetate
3-Decanone	9	50	95	Heptyl propionate and ethyl caprylate (1:1) <sup>d</sup>
4-Decanone	12	62	100	Hexyl butyrate
2-Undecanone	5	3	35	Nonyl acetate and methyl decanoate (7:2) <sup>e</sup>
2-Dodecanone	2.5	0	5	Decyl acetate and methyl undecanoate (7:2) <sup>f</sup>
2-Tridecanone	0	6	6	Methyl laurate

n.d. = Not determined

<sup>a</sup> 50 mM substrate, 200 mM NADPH

<sup>b</sup> 10 mM substrate

<sup>c</sup> 10 mM substrate, 20 mM glucose for cofactor regeneration

<sup>d</sup> Crude cell extract: heptylpropionate only; growing cells: heptylpropionate and ethylcaprylate (1:1)

<sup>e</sup> Crude cell extract: nonylacetate only; growing cells: nonylacetate and methyldecanoate (5:2)

<sup>f</sup> Crude cell extract: decylacetate only

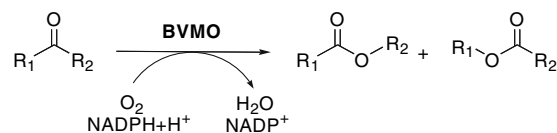
All values were measured after 8 h at pH 7.5 and 25°C

or resting, revealed that the latter system worked more efficiently at all investigated temperatures since conversions were higher and some ketones were oxidized even quantitatively. In the case of crude cell extract, sonication leading to the loss of the enzyme protecting physiological environment can influence enzyme activity and stability. This might be a possible explanation for reduced conversions. When using growing cells, substrate was added simultaneously to the time-point of induction of BVMO expression, whereas for resting cells the enzyme was first expressed before starting biotransformations. Conclusively, the reaction times for growing cells are much longer compared to resting cells. Another problem using growing cells was that 2-nonanone (or an unknown impurity in the substrate) seemed to have a harmful effect, since no conversion could be detected and no more growth could be observed after substrate addition. Thus, resting cells seemed to give best results working sufficiently and cost-effective.

Interestingly, conversion of some ketones did not only generate one ester product. According to Fig. 2, oxygen can be inserted on both sides of the keto group producing alkylacetates as well as the less favored methyl esters. With longer chain length the enzyme does not insert oxygen by migration of the

higher substituted alkyl chain as typical for Baeyer-Villiger reactions. Instead, conversion into the methylesters seem to be preferred. Comparing these results to those of the BVMO from *Ps. fluorescens* DSM50106 it strikes that for this enzyme the migrating substituent during oxygen insertion depends on the position of the keto group. While here all 2-ketones became acetates, 3-ketones were converted into both ester products and 4-ketones gave propylesters only (unpublished results). In the case of EtaA from *Mycobacterium tuberculosis* insertion of oxygen depends on alkyl chain length and position of the keto group (Fraaije et al. 2004).

Examining the different results at 20, 25 and 30°C also revealed a correlation between reaction temperature and conversion. Biotransformations at 30°C using resting cells lead to a two-fold increase or quantitative conversion in comparison to 20°C after



**Fig. 2** BVMO catalyzed conversion of aliphatic open-chain ketones

the same reaction time (data not shown). At higher temperatures reaction times were shorter but, especially for the short-chain ketones, the effect of evaporation was also more evident.

## Conclusion

In this study, the gene of a Baeyer-Villiger monoxygenase from *Pseudomonas putida* KT2440 was successfully amplified from genomic DNA and cloned into two different vectors. Performing coexpression of different chaperones with the BVMO successfully lead to soluble protein. Investigation of substrate specificity revealed a high preference towards short-chain aliphatic ketones. Comparing biocatalysis using crude cell extract and whole cells showed that resting cells worked most efficiently and gave the best results. Additionally, it was found that some acyclic ketones were converted not only to the alkylacetates, but also methyl- and ethylesters were obtained, indicating insertion of oxygen on both sides of the keto group.

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## References

- Britton LN, Markovetz AJ (1977) A novel ketone monoxygenase from *Pseudomonas cepacia*. *J Biol Chem* 252:8561–8566
- Donoghue NA, Norris DB, Trudgill PW (1976) The purification and properties of cyclohexanone oxygenase from *Nocardia globberula* CL1 and *Acinetobacter calcoaceticus* NCIMB 9871. *Eur J Biochem* 63:175–192
- Fraaije MW, Kamerbeek NM, Heidekamp AJ et al (2004) The produg activator EtaA from *Mycobacterium tuberculosis* is a Baeyer-Villiger monoxygenase. *J Biol Chem* 279:3354–3360
- Fraaije MW, Kamerbeek NM, van Berkel WJH et al (2002) Identification of a Baeyer-Villiger monoxygenase sequence motif. *FEBS Lett* 518:43–47
- Kamerbeek NM, Moonen MJH, van der Ven JGM et al (2001) 4-Hydroxyacetophenone monoxygenase from *Pseudomonas fluorescens* ACB. *Eur J Biochem* 268:2547–2557
- Kirschner A, Altenbuchner J, Bornscheuer UT (2007) Cloning, expression and characterization of a Baeyer-Villiger monoxygenase from *Pseudomonas fluorescens* DSM 50106 in *E. coli*. *Appl Microbiol Biotechnol* 73:1065–1072
- Kirschner A, Bornscheuer UT (2006) Baeyer-Villiger monoxygenase-catalyzed kinetic resolution of 4-hydroxy-2-ketones. *Angew Chem Int Ed* 45:7004–7006
- Lee DH, Kim MD, Lee WH et al (2004) Consortium of fold-catalyzing proteins increases soluble expression of cyclohexanone monoxygenase in recombinant *Escherichia coli*. *Appl Microbiol Biotechnol* 63:549–552
- Malito E, Alfieri A, Fraaije MW et al (2004) Crystal structure of a Baeyer-Villiger monoxygenase. *Proc Natl Acad Sci USA* 101:13157–13162
- Mihovilovic MD, Müller B, Stanetty P (2002) Monoxygenase-mediated Baeyer-Villiger oxidations. *Eur J Org Chem* 2002:3711–3730
- Nelson KE, Weinel C, Paulsen IT et al (2002) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* 4:799–808
- Stewart JD (1998) Cyclohexanone monoxygenase: a useful reagent for asymmetric Baeyer-Villiger reactions. *Curr Org Chem* 2:211–232
- Tanner A, Hopper DJ (2000) Conversion of 4-hydroxyacetophenone into 4-phenyl acetate by a flavin adenine dinucleotide-containing Baeyer-Villiger-type monoxygenase. *J Bacteriol* 182:6565–6569