Chapter 5

A generic, whole-cell-based screening method for Baeyer–Villiger monooxygenases

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Abstract

Baeyer–Villiger monooxygenases (BVMOs) receive increasing attention as enzymes useful for biocatalytic applications. Industrial requirements call for rapid and extensive redesign of these enzymes. In response to the need of screening large libraries of BVO mutants, we have established a generic screening method that allows screening *Escherichia coli* cells expressing BVMOs in 96-well plate format. For this, we first developed an expression system for production of phenylacetone monooxygenase (PAMO) in the periplasm of *E. coli*. This allows probing the enzyme for any target substrate while it is also compatible with extracellular coenzyme regeneration. For coenzyme regeneration, we used phosphite dehydrogenase which forms phosphate upon NADPH recycling. This allowed usage of a chromogenic molybdate-based phosphate determination assay. The screening procedure was supplemented with a detection method for identification of mutant enzymes that act as NADPH oxidases, thereby excluding false positives. The whole-cell-based screening method was validated by screening site-saturation libraries of PAMO and resulted in the identification of PAMO mutants with altered catalytic properties. This new method can be used for screening libraries of BVMOs for activity with any desired substrate and, therefore, is a powerful tool for engineering of these enzymes.
Introduction

Baeyer–Villiger monooxygenases (BVMOs) are promising enzymes with respect to industrial applications as they offer the possibility to perform, among others, oxygen atom insertion into a C–C bond and enantioselective sulfoxidations (Balke et al., 2012; Leisch et al., 2011). However, the properties of naturally occurring enzymes are typically far from the demands set by commercial processes. Features such as substrate acceptance, stereoselectivity, and stability need to be improved before these enzymes can be applied on industrial scale. Directed evolution is a powerful tool for engineering enzymes. Despite the potential significance of BVMOs, there have been only few examples of directed evolution experiments on this class of enzymes. In most of the reported studies, screening for BVMO variants with improved properties was performed using chromatographic methods such as GC (Clouthier et al., 2006; Reetz et al., 2004a; Reetz and Wu, 2008; Reetz and Wu, 2009; Wu et al., 2010) or HPLC (Reetz et al., 2004b). Unfortunately, these screening methods are of limited throughput, demand establishing optimal separation conditions for each targeted compound, and require specialised equipment. In addition, the adrenalin assay was applied in screening for enantioselective variants of a BVMO from Pseudomonas fluorescens DSM 50106 (Kirschner and Bornscheuer, 2008). In this assay, the hydrolysis of Baeyer–Villiger products yielded 1,2-diols, which were then quantified in a colorimetric assay. Furthermore, Bornscheuer and co-workers used p-nitroacetophenone to detect 4-hydroxyacetophenone mono-oxygenase activity (Saß et al., 2012). In this assay, Baeyer–Villiger oxidation of the substrate and hydrolysis of the product lead to the formation of p-nitrophenolate. The assay was applied in screening libraries of 4-hydroxyacetophenone mono-oxygenase mutants yielding variants with improved activity towards p-nitroacetophenone. However, the latter two methods can only be used for specific substrates.

A few other methods for screening BVMOs have been described, for instance, a pH-change-based assay (Watts et al., 2002). This assay revolves around hydrolysis of the Baeyer–Villiger product, resulting in the release of acid. However, this assay requires extensive optimisation to give reliable signals and an esterase specific for the Baeyer–Villiger product. Furthermore, several fluorogenic methods, employing umbelliferone-labelled substrates, have been used successfully for the detection of BVMOs (Gutiérrez et al., 2003; Sicard et al., 2005). The underlying principle of the latter methods is the Baeyer–Villiger oxidation of the umbelliferone-labelled substrate and subsequent hydrolysis of the Baeyer–Villiger product, resulting in the release of the fluorescent umbelliferone. A screening method for ketone biotransformations was also presented, which was used successfully for the detection of BVMO activity (Linares-Pastén et al., 2012). This method is based on the reaction between alicyclic ketones and 3,5-dinitrobenzoic acid under alkaline...
conditions, resulting in the formation of a purple coloured product. Despite being fast and simple, this method requires relatively high activities, and it is limited to a certain class of compounds.

Although a variety of screening methods for the detection of BVMO activity has been described (see above), most of these methods have never been tested in BVMO-mutant-library screening, are of limited throughput, are designed for specific substrates, or require the synthesis of labelled substrates. The drawbacks of the current screening methods underscore the need of a generic, preferably high-throughput, screening method for BVMO activity. Here we report on a screening method for NAD(P)-dependent enzymes, in particular BVMOs (Figure 1). This screening system was validated for phenylacetone monooxygenase from *Thermobifida fusca* (PAMO, EC 1.14.13.92, Fraaije et al., 2005). We expressed PAMO in the periplasm of *Escherichia coli* to ensure the unlimited access of substrates to the enzyme and to enable the shuttling of nicotinamide coenzymes between PAMO and externally added phosphite dehydrogenase (PTDH). The latter enzyme is required for NADPH regeneration (Torres Pazmiño et al., 2008). Coenzyme regeneration by PTDH results in phosphate as a side-product, which

![Figure 1](image-url)

**Figure 1.** Schematic representation of the principle of the developed screening method. PAMO is translocated to the periplasm by the Tat transporter. In the periplasm, PAMO performs the oxidation of a substrate at the expense of NADPH. NADPH and NADP⁺ can freely diffuse into and out of the periplasm. PTDH added to the reaction medium reduces NADP⁺ to NADPH while oxidising phosphite to phosphate. The concentration of the latter compound is measured in the reaction with molybdate by forming a blue product. Hydrogen peroxide, a product of the uncoupling reaction, is detected by fluorogenic Peroxy Green 1 (IM, inner membrane; OM, outer membrane).
can be used as a reporter of Baeyer–Villiger activity. To quantify phosphate, we used a modified version of the molybdate assay (Saheki et al., 1985), which allows colorimetric detection. To increase the fidelity of our screening procedure, we included a control reaction to exclude false positive clones. To this end, we detect hydrogen peroxide, which is typically produced during uncoupling of the BVMO reaction at the expense of NADPH and oxygen (Torres Pazmiño et al., 2008). The integration of periplasmic expression of PAMO, coenzyme regeneration, and spectrophotometric detection of coupled products resulted in an accurate and reliable screening method, which can be applied to test monooxygenase activity on any desired substrate.

Materials and Methods

Enzymes, reagents, and strains. Unless indicated, all chemicals were obtained from Sigma-Aldrich, Acros Organics, TCI Europe, Roche Diagnostics GmbH, and Merck. *PfuTurbo* was purchased from Stratagene (La Jolla, CA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). In-Fusion 2.0 Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA) was used for DNA cloning. dNTPs were ordered at Clontech. Nucleotide primers were purchased at Sigma. NADPH was purchased at Oriental Yeast Co. Ltd. (Tokyo, Japan). Sodium phosphite was obtained from Riedel de Haën (Seelze, Germany). Peroxy Green 1 was kindly provided by P. Wiererinck (Organon, The Netherlands). *E. coli TOP10* cells were obtained from Invitrogen. DNA sequencing was performed at GATC Biotech (Konstanz, Germany). A modified low-phosphate AP5 medium (Chang et al., 1987) was used to grow *E. coli* cells expressing Tat-PAMO. The AP5 medium contained: NaCl (60 mM), KCl (10 mM), NH₄Cl (20 mM), MgSO₄ (1.6 mM), triethanolamine (150 mM, pH 7.4), glycerol (0.15% (v/v)), and N-Z-amine A from bovine milk (0.3% (w/v), Fluka). As compared to the original recipe, glucose was replaced by glycerol, and no phosphate source was added. ~0.3 mM phosphate present in the medium comes from casein hydrolysate N-Z-amine A. All reagents used in the screening were prepared using filtered water. Molybdate reagent was prepared by dissolving zinc acetate (100 mM, Fluka) and ammonium molybdate ((NH₄)₆Mo₇O₂₄•4H₂O, 10 mM, Sigma) and adjusting pH to 5.0. The solution was stored at room temperature in a plastic bottle and was stable for at least six months. Ascorbic acid solution was prepared by dissolving L- (+)- ascorbic acid (10% (w/v), Sigma-Aldrich) and adjusting pH to 5.0. It was aliquoted and stored at –20 °C. Stock solutions of phenylacetone (1 mM), benzyl phenyl sulfide (0.125 mM), and Peroxy Green 1 (25 mM) were prepared by dissolving the respective compounds in 1,4-dioxane.

Subcloning, expression, and purification of PTDH. A codon-optimised thermostable variant of the *ptdx* gene was amplified from the pCRE2 vector (Torres Pazmiño et al., 2009) and subcloned into the same vector using NdeI/HindIII sites. This yielded the pPTDH plasmid in which *ptdx* gene is fused to an N-terminal His-tag. *E. coli TOP10* cells were transformed with this plasmid and grown in LB supplemented with ampicillin (50 μg mL⁻¹, LB Amp). The next day the cells were diluted (1:100) in Terrific Broth (TB) supplemented with ampicillin (50 μg mL⁻¹) and L-arabinose (0.02% (w/v)) and incubated at 30 °C for 16 h. The protein was purified using Ni Sepharose High Performance (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Purified PTDH was flash-frozen with the addition of glycerol (10% (v/v)) and stored at –80 °C. The concentration of PTDH was determined using two assays: Waddell’s method (Waddell, 1956) and the estimated extinction coefficient at 280 nm (calculated using the ProtParam tool, http://web.expasy.org/protparam): \( \varepsilon_{280\,\text{nm}} = 26.5 \, \text{mm}^{-1} \, \text{cm}^{-1} \).

Construction of mutants and mutant libraries. Mutants of Tat-PAMO used as controls in the screening (R337A, M446G, Q152F, Q152F/L153G/M446G), as well as the site-saturation libraries I67X and M446X were constructed by QuikChange PCR using standard mutagenic primers and
following the manufacturer’s recommendation. The libraries were constructed by introduction of the NNK codon at the respective positions.

**Export of PAMO to the periplasm.** The *pamO* gene was cloned into the previously described pBAD-Tat-AldO plasmid (van Bloois et al., 2009) via EcoRI and HindIII sites, replacing the *aldO* sequence in the vector. In this way, the *pamO* gene was fused to an N-terminal signal sequence from *E. coli* TorA protein and a C-terminal Myc epitope/His-tag. *E. coli* TOP10 cells were transformed with the Tat-PAMO plasmid. In order to test whether PAMO was present in the periplasm, the cells harbouring Tat-PAMO plasmid were grown in LB$_{Amp}$ supplemented with L-arabinose (0.0002–0.005% (w/v)) at 17 °C or 24 °C for 48 h and at 37 °C for 16 h, and subsequently fractionated with the help of PeriPreps PeriPlasting kit (Epicentre Biotechnologies, Madison, WI). Specific activity of PAMO in spheroplast and periplasmic fractions was measured using the NADPH depletion activity assay while Bradford assay (Bio-rad, Hercules, CA) was employed to assess the protein content in the samples. In the NADPH depletion assay, the reaction mix contained phenylacetone (1 mM), NADPH (100 µM), and DMSO (1% (v/v)). The reaction was followed by measuring the absorbance at 340 nm ($\varepsilon_{\text{NADPH, 340 nm}} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1}$).

**Expression of Tat-PAMO in 96-well plates.** All optical measurements (optical density, OD$_{600}$; absorbance; fluorescence) were conducted on a SynergyMx micro-titer plate reader (BioTek Instruments, Inc., Winooski, VT) using appropriate plates purchased from Greiner Bio-One (Kremsmünster, Austria). Cells were routinely grown in 2 mL 96-square well plates (Waters, Milford, MA) covered with air-permeable AeraSeal Sealing Films (Excel Scientific, Victorville, CA). The plates were incubated in a Titramax 1000 shaker (Heidolph, Schwabach, Germany) at 1050 rpm. Pre-cultures were inoculated from master plates using a cryo-replicator (Enzyscreen, Haarlem, The Netherlands) into a 96-well plate containing AP$_5$$_{Amp}$ (1 mL) medium in each well. The plate was incubated at 30 °C for ~22 h. Afterwards, 60 µL of each pre-culture was diluted in fresh AP$_5$$_{Amp}$ (800 µL) pre-warmed to 37 °C and incubated at 37 °C for 110–120 min until the cultures reached the OD$_{600}$ of 0.4–0.5. Then L-arabinose (40 µL of 4% (w/v) solution) was added to the cultures along with FAD (9 µL of 1 mM solution). The plate was incubated at 37 °C for 16 h.

**Screening procedure.** After overnight expression of Tat-PAMO, cells were pelleted by centrifugation for 20 min at 2250 × g at 4 °C and resuspended in reaction mixture (250 µL) which contained: NADPH (20 µM), sodium phosphate (20 mM), 1,4-dioxane (1% (v/v)), sodium cyanide (20 mM), Peroxy Green 1 (250 µM), PTDH (5 µM), FAD (10 µM), Tris-HCl (50 mM, pH 9.0), and glycerol (10 mM). Afterwards, 10 µL of benzyl phenyl sulfide (0.125 M) was dispensed into each well. In the case of activity assays with phenylacetone, the reaction mixture contained: substrate (10 mM), NADPH (20 µM), sodium cyanide (20 mM), Peroxy Green 1 (250 µM), PTDH (5 µM), FAD (10 µM), Tris-HCl (50 mM, pH 7.5), and glycerol (10 mM). Reactions with phenylacetone were conducted for 1.5 h at 37 °C, 1050 rpm. In the case of benzyl phenyl sulfide, the reaction time was 1.5 or 3 h. Subsequently, the plate was centrifuged for 5 min at 2250 × g at 4 °C. 100 µL of the supernatant was used to measure the fluorescence. Excitation was at 460 nm, and emission was recorded at 510 nm while the sensitivity was set to 45. Mutants presenting at least 80% of the fluorescence emitted by the Q152F/L153G/M446G mutant were regarded as the uncoupling mutants and not considered for further analysis. For the quantification of phosphate, the supernatant (20 µL) was mixed with the molybdate reagent (200 µL), followed by the addition of ascorbic acid (50 µL). After 30 min incubation at 30 °C and 900 rpm, absorbance was read at 850, 700, and 600 nm. Phosphate standards containing potassium dihydroxy phosphate (from 0.2 to 10 mM) were used to prepare the calibration curve for the molybdate assay. The $Z'$-factor was used as a measure of the quality of the assay during the optimisation, and it was calculated according to the formula introduced by Zhang et al. (1999):

$$Z' = 1 - \frac{3\sigma_+ + 3\sigma_-}{|\mu_+ - \mu_-|}$$

in which $\mu_+$ and $\mu_-$ denote means of positive and negative control, respectively, and $\sigma_+$ and $\sigma_-$ denote standard deviations.
**Whole-cell activity measurements.** In order to evaluate the phosphate as a reporter of the BVMO activity, the Baeyer–Villiger product formed by Tat-PAMO cells was quantified by means of GC. To this end, wild-type and R337A Tat-PAMO cells were grown in 96-well plates and the protein expression was induced with l-arabinose. Harvested cells were resuspended in Tris-HCl (50 mM, pH 7.5) with glycerol (10 mM) and transferred to 2 mL glass screw cap vials (Brown Chromatography Supplies, Wertheim, Germany). Then phenylacetone and other reaction mixture components were added to the vials at the same concentrations as used in the screening, except that in half of the samples, PTDH was replaced with the buffer. After 90 min conversion at 37 °C, vials were spun down. 20 µL of the supernatant was used for the phosphate determination in the molybdate assay, while 200 µL of the supernatant were extracted with tert-butyl methyl ether (400 µL), dried over MgSO₄ and separated on an AT5 column (30 m × 0.25 mm × 0.25 µm, Grace, Deerfield, IL) using the following temperature program: 2 min at 100 °C, 5 °C/min up to 120 °C, 20 °C/min up to 160 °C. Retention times of phenylacetone and benzyl acetate were 6.96 min and 7.45 min, respectively.

**Characterisation of the mutants.** Plasmids of clones selected in the first round of screening were isolated. The plasmid DNA was used to transform fresh *E. coli* TOP10 cells, and the new clones were retested using the same assay. Clones with confirmed activity were expressed in TB (50 mL) and the proteins were purified using Ni Sepharose High Performance. 1 mL reactions containing PAMO mutant (4 µM), PTDH (4 µM), NADPH (200 µM), benzyl phenyl sulfide (5 mM), sodium phosphite (20 mM), Tris-HCl (50 mM, pH 9.0) were incubated at 30 °C for 48 h. Afterwards, they were extracted with ethyl acetate, dried over MgSO₄ and analysed by GC on an HP1 column (30 m × 0.32 mm × 0.25 µm, Agilent, Santa Clara, CA) using the following temperature program: 5 min at 100 °C, 5 °C/min up to 200 °C, 5 min at 200 °C. Retention times were 20.9 min, 25.6 min, and 26.2 min for the substrate, the sulfoxide, and the sulfone, respectively. The enantiomeric excess (ee) of the product was estimated by evaporating the samples, dissolving them in n-heptane/isopropanol (9:1) and separating in chiral HPLC on a Chiralcel OD column (0.46 cm × 25 cm, Daicel, Japan) using isocratic n-heptane/isopropanol (9:1), flow 0.6 mL/min, 45 min, 40 °C. The (R)-enantiomer was eluted at 29 min and the (S)-sulfoxide at 32 min. ThermoFAD measurements were performed as described before (Dudek et al., 2011).

**Results**

*Expression of PAMO in the periplasm*

We sought to develop a generic screening procedure for NAD(P)-dependent enzymes based on whole cells, using PAMO as a model BVMO. In order to be successful, this system should offer unrestricted access of the enzyme towards the substrate tested. Unfortunately, many (potential) substrates are unable to cross the cytoplasmic membrane and, therefore, cannot react with the target enzyme expressed in the cytoplasm. Several strategies have been applied to increase substrate accessibility. Secretion of the target enzyme out of the cytoplasm to the periplasm is one of the typical approaches. The Tat pathway is the only one among bacterial translocation system that is capable of transporting fully folded and often cofactor-containing proteins to the periplasm and is, therefore, of considerable biotechnological importance (Lee et al., 2006). Considering that PAMO requires non-covalently bound FAD cofactor for its activity, we decided to study whether the Tat pathway could be employed for functionally transporting PAMO to the periplasm. To this end, PAMO was N-terminally fused to the Tat-dependent signal
sequence of the endogenous *E. coli* protein TorA (Weiner et al., 1998), resulting in Tat-PAMO. Subsequently, we determined the best conditions for periplasmic expression of Tat-PAMO. For this, Tat-PAMO was expressed in *E. coli* under control of an arabinose-inducible promoter using increasing amounts of arabinose at 17 °C, 24 °C, or 37 °C. These cells were harvested and fractionated into spheroplast and periplasmic fraction. Of note, the spheroplast fraction contains the cytoplasmic and total membrane fractions. Next, we analysed the specific activities of these fractions using phenylacetone as a substrate (Figure 2). At 17 °C and 24 °C, the highest activity towards phenylacetone was clearly observed in the spheroplast fraction relative to the periplasmic fraction regardless of the arabinose concentration. However, at 37 °C the differences in the observed activities between the different fractions became less pronounced. In particular, a slightly higher specific activity for phenylacetone was observed in the periplasmic fraction derived from cells induced for Tat-PAMO expression with 0.0002% arabinose relative to the spheroplast fraction prepared from the same cells. This indicates that PAMO was indeed for a significant part translocated to the periplasm.

Although our data show that attachment of the TorA signal sequence does not inactivate PAMO, it cannot be excluded that this compromises the enzyme to some extent. Therefore, we decided to analyse the properties of PAMO isolated from a periplasmic preparation in more detail. Firstly, we found that the enzyme obtained from a periplasmic preparation runs at the same position in SDS-PAGE as native PAMO expressed in the cytoplasm, indicating that the TorA signal sequence is proteolytically removed during periplasmic export as shown for other Tat-dependently exported proteins (Lee et al., 2006). Secondly, the $k_{cat}$ and $K_M$

![Figure 2](image-url). Specific activity of periplasmic and spheroplast fractions of PAMO towards phenylacetone. *E. coli* cells expressing Tat-PAMO were fractionated into periplasm and spheroplast fractions. The concentrations of arabinose and temperatures used for the expression of Tat-PAMO are detailed.
values for phenylacetone of PAMO expressed in the periplasm did not differ from the kinetic parameters of wild-type PAMO. Lastly, the apparent melting temperature of the mature-sized periplasmic form measured by the ThermoFAD method (Forneris et al., 2009) was the same as of wild-type PAMO (60 °C). This confirms that the function of PAMO is not affected by fusing it to the TorA signal sequence.

**Culture conditions**

Subsequent experiments aimed at optimising the conditions for the periplasmic export of PAMO. To this end, we developed an activity-based assay in 96-well plate format revolving around phosphate detection. After evaluating different growth media for our screening method, we obtained the best results with a modified version of AP5, a defined, low-phosphate medium (Chang et al., 1987). The phosphate content in this medium was found to be only ~0.3 mM compared to, for example, ~4 mM in LB medium. The low amount of phosphate in AP5 ensures low background signals without the need of washing cells before the activity assay. Importantly, the growth of *E. coli* is not severely inhibited by the limited amount of phosphate in liquid AP5 medium as evidenced by the optical density of 2.5–3 when cells are grown to saturation in AP5 (Figure 3A).

In order to effectively control the growth of cells in individual wells, the optimised protocol involved growing pre-cultures inoculated with cells from a glycerol stock or fresh transformants. The pre-cultures were then diluted in fresh AP5 medium and grown for a given time before arabinose was added to induce the production of Tat-PAMO. Previously, we assessed several critical factors that control the reproducible expression of PAMO, and we observed that the cellular growth stage at which induction is initiated is of crucial importance for good overexpression (van Bloois et al., 2012). Therefore, we also determined the best induction time for the expression of Tat-PAMO. Furthermore, in the case of periplasmic expression, the amount of the protein in the periplasm depends not only on the overall expression level but also on the efficiency of the export to the periplasm. In other words, the highest total expression level may not correspond to the highest periplasmic expression. Therefore, we evaluated the induction protocol by measuring the whole-cell activity of Tat-PAMO cells with the phosphate assay. The highest activity was observed when cells were induced during the early exponential phase (Figure 3B). Since we observed a clear peak in activity depending on the growth phase, care had to be taken in order to induce the cells always in the same growth phase. We achieved this by using high volume inocula, medium pre-warmed to 37 °C, and precise culturing times.

We analysed whether external addition of FAD would improve the activity of the enzyme because it cannot be excluded that the enzyme partially loses its cofactor when it is placed in the environment lacking any free FAD such as the periplasm.
Figure 3. (A) Growth curve of *E. coli* cells transformed with the Tat-PAMO expression plasmid grown in AP5 medium in 96-well plate format. (B) The activity of the cells expressing Tat-PAMO depends strongly on the growth phase at which the protein expression is induced. Each point is an average of four measurements. Error bars indicate standard deviation. (C) Phosphate determination in samples of the inactive mutant Tat-PAMO R337A (0.4 mM phosphate) and wild-type Tat-PAMO (3.5 mM phosphate) after the reaction with phenylacetone.

Therefore, we tested several conditions in which 10 or 100 µM FAD was added to the culture medium during the expression and/or to the reaction medium during the biotransformation. Surprisingly, we observed that the activity of Tat-PAMO can be doubled by adding 10 µM FAD during both the expression and the biotransformation. This indeed suggests that PAMO partially loses FAD in the periplasm.
Coenzyme regeneration system

We have previously reported on an elegant system for coenzyme regeneration suitable for BVMOs by fusing them to PTDH (Torres Pazmiño et al., 2008; Torres Pazmiño et al., 2009). We sought to use the same coenzyme regeneration system for the design of our screening method. Under normal conditions, namely no phosphate limitation, phosphite does not enter the cytoplasm of E. coli because pathways for assimilation of alternative phosphorus forms are repressed (Metcalf and Wanner, 1991; Wanner, 1993). However, under these conditions, phosphite is most likely able to cross the outer membrane and enter the periplasmic space. Export of PTDH to the periplasm is, therefore, required to enable efficient cofactor recycling in this cellular compartment. However, appending PTDH to the N-terminus of Tat-PAMO impaired Tat-mediated transport of this fusion protein (data not shown). Therefore, we decided to use the established periplasmic expression of PAMO in combination with externally added PTDH since NADP(H) can diffuse into and out of the periplasm through the outer membrane pores. To facilitate overexpression and purification, PTDH was sub cloned into the pBAD vector with an N-terminal His-tag. It could be purified on Ni²⁺-resin yielding up to 600 mg of protein per 1 L medium. In this way, the export of PAMO was not compromised by addition of the fusion partner. Furthermore, by adding isolated PTDH to the reactions, the concentration of the enzyme can be manipulated.

Phosphate detection in molybdate assay

Methods commonly used for quantification of inorganic phosphate employ ammonium molybdate that reacts with phosphate to form a chromophore. These methods are typically modifications of a procedure published by Fiske and Subbarow (1925), and they require the use of strong acids which is not compatible with biological samples. Therefore, we adapted a method of Saheki et al. which was developed for assaying biological samples and is performed in slightly acidic conditions (Saheki et al., 1985). In this method, the phosphomolybdate complex is reduced by ascorbic acid in the presence of Zn²⁺ ions, which leads to the formation of a blue product with an absorbance maximum at around 850 nm. As compared to the protocol of Saheki et al., we decreased the ammonium molybdate concentration from 15 to 10 mM and increased the time of incubation at 30 °C from 15 to 30 min. The colour formed is stable for at least 1 h. We down-scaled the procedure to make it compatible with 96-well plate format. A linear dependence between the phosphate concentration and the absorbance was observed up to 4 mM phosphate. For phosphate concentrations higher than 4 mM, the absorbance exceeded the detection limit. By shifting the detection wavelength to 600 nm, we extended the working range of the assay up to 10 mM phosphate. If required,
active and inactive clones can be distinguished by direct visual inspection, as presented in Figure 3C.

**Control of the uncoupling**

It has been observed for BVMOs that in the absence of a suitable substrate, the peroxyflavin intermediate can decay forming hydrogen peroxide (Torres Pazmiño et al., 2008). In this way, a BVMO can act as an NADPH oxidase. This process, referred to as uncoupling, is unfavourable in our screening for two reasons. First, mutants with high uncoupling rate may give high signals in the screening while they perform the desired conversion at low rate or are completely inactive in the Baeyer–Villiger reaction. Thus, uncoupling may result in the isolation of false positive clones in our NADPH-consumption-based screening system. Second, enzymes presenting increased uncoupling rates, even when they are still able to catalyse the reaction, work less efficiently because of the elevated coenzyme requirements and often reduced catalytic rates. Meanwhile, mutations introduced in the active site of the enzyme in order to alter its catalytic properties may affect the stability of the peroxyflavin, thereby increasing the uncoupling rate. These reasons prompted us to include hydrogen peroxide detection in order to increase the fidelity of our method. To this end, we decided to test Peroxy Green 1. It has been established that this dye is a highly specific probe for hydrogen peroxide (Miller et al., 2007). In the presence of hydrogen peroxide, the boronate moiety is cleaved off leading to the formation of fluorescent 2-methyl-4-O-methyl Tokyo Green (Figure 4).

![Peroxy Green 1](image)

For establishing the conditions of the fluorescence measurements, we selected the previously characterised Q152F PAMO mutant (Dudek et al., 2011) and the triple Q152F/L153G/M446G PAMO mutant (Torres Pazmiño et al., 2007). Both mutants exhibit high uncoupling rates (0.5–0.6 s$^{-1}$) relative to wild-type PAMO (0.02 s$^{-1}$, Torres Pazmiño et al., 2008). These mutations were subsequently introduced in Tat-PAMO. We found out that the cells producing these Tat-PAMO variants show Peroxy Green 1 based fluorescence values higher than wild-type

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**Figure 4.** Hydrogen peroxide reacts with Peroxy Green 1 leading to fluorescent 2-methyl-4-O-methyl Tokyo Green.
Tat-PAMO only if catalase activity is inhibited by adding sodium cyanide to the reaction medium. Conceivably, the increased amounts of hydrogen peroxide produced in these cells induce the expression of catalase activity. Nevertheless, in the presence of Peroxy Green 1 and sodium cyanide, a clear increase in the fluorescence emitted by the uncoupling mutant as compared to wild-type PAMO was observed. Furthermore, addition of Peroxy Green 1 or sodium cyanide did not interfere with the phosphate detection. These findings show that Peroxy Green 1 can be incorporated in the phosphate screening method for efficient hydrogen peroxide detection, which allows elimination of variants with high uncoupling rates.

Evaluation of the screening method

After having established the best conditions for the expression of Tat-PAMO and the conditions for our screening method, we tested whether this optimised protocol could be used in a reproducible way to distinguish cells expressing the previously described PAMO R337A mutant from cells expressing wild-type PAMO, using phenyl acetone as substrate. The R337A mutant is inactive in Baeyer–Villiger reaction and consumes NADPH at a very low rate \(0.001 \text{ s}^{-1}\) (Torres Pazmiño et al., 2008). To this end, we inoculated 32 wells with single colonies of *E. coli* cells transformed with a Tat-PAMO expression plasmid and 32 wells with cells harbouring a Tat-PAMO-R337A expression plasmid. Each of these pre-cultures was subsequently used to inoculate an expression culture which was then assayed for BVMO activity as described in Materials and Methods. The amounts of phosphate produced by each culture are shown in Figure 5A. In the control reaction of wild-type PAMO, the mean signal of 5.0 mM was obtained while the background signal given by the inactive mutant R337A was only 0.54 mM (Table 1). This background signal can be caused by NADPH-consuming enzymes released from lysed cells. Relative standard deviations calculated from these data did not exceed 12%, which is a satisfactory value for a whole-cell-based system. Furthermore, these data correspond to a \(Z'\)-factor value of 0.6 which indicates very good separation of the signals and confirms the reliability of our screening method (Zhang et al., 1999).

Encouraged by these results, we used the same experimental set-up to test whether our method could be used to distinguish cells expressing the PAMO mutant M446G from cells expressing the R337A variant using benzyl phenyl sulfide as a substrate. This prochiral bulky compound is not converted by wild-type PAMO while the M446G mutant has been shown to oxidise it to the corresponding sulfoxide (Dudek et al., 2011). In order to compensate for the low conversion rate, the reaction was performed at pH 9.0, at which the activity of PAMO is increased relative to pH 7.5. 1,4-dioxane at a concentration of 6% (v/v) was present in the reaction mixture in order to facilitate the solubilisation of the substrate. This amount of organic solvent did not inactivate either PAMO or
PTDH. The reaction was conducted for 90 min. As shown in Figure 5B, the signals given by the M446G mutant can be clearly distinguished from the background signals. Not surprisingly, the separation band is smaller than in our previous control reaction of the wild-type enzyme with phenylacetone, but this reaction reflects better the situation of screening for activity on a challenging substrate. The relative standard deviations were at the level of 11–12% while the calculated $Z'$-factor value was 0.3 (Table 1), indicating that the method still allows accurate screening.

**Figure 5.** Phosphate signals obtained in independent replicates of the reaction of (A) wild-type PAMO and the R337A mutant with phenylacetone and (B) the M446G and R337A mutants with benzyl phenyl sulfide. The data points are plotted in descending order.
In order to verify whether the measured phosphate levels correspond to the actual product formation, we employed GC for the quantification of the Baeyer–Villiger product in parallel with phosphate measurements. Due to the adsorption of both phenylacetone and benzyl acetate onto plastic, we used glass vials mimicking the conditions in wells of a 96-well plate. 5.0 ± 0.8 mM of benzyl acetate was produced by Tat-PAMO cells in the presence of the coenzyme regeneration system as determined by GC analysis. Control experiments in the absence of the regeneration system showed that only traces of benzyl acetate (< 0.1 mM) were detected. This indicates that Baeyer–Villiger activity of Tat-PAMO cells is related to the periplasmic fraction of the enzyme, which is dependent on the external coenzyme regeneration. When the same samples were analysed using the phosphate detection assay, 5.4 ± 1.2 mM of phosphate was measured. Only 0.6 ± 0.2 mM of phosphate is produced by the R337A variant in this reaction, while no oxidised product was detected by GC analysis. This indicates that coenzyme recycling drives the production of about 4.8 mM phosphate by a BVMO, demonstrating the effectiveness of our coenzyme regeneration system. Thus, the obtained values of phosphate and benzyl acetate production under the assay conditions are within 10% of each other. These results confirm the applicability of phosphate as a reporter of BVMO activity in our system.

Table 1. Reproducibility of the screening method. Phosphate levels (mM) obtained for positive and negative control strains in reactions with phenylacetone and benzyl phenyl sulfide are presented (SD, standard deviation; RSD, relative standard deviation).

<table>
<thead>
<tr>
<th></th>
<th>phenylacetone</th>
<th>benzyl phenyl sulfide</th>
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<tbody>
<tr>
<td></td>
<td>wild-type</td>
<td>R337A</td>
</tr>
<tr>
<td>mean</td>
<td>5.0</td>
<td>0.54</td>
</tr>
<tr>
<td>SD</td>
<td>0.52</td>
<td>0.06</td>
</tr>
<tr>
<td>RSD</td>
<td>10%</td>
<td>11%</td>
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<tr>
<td>Z'</td>
<td>0.6</td>
<td>0.3</td>
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Screening libraries of PAMO

The ability of our screening method to distinguish active PAMO variants from inactive ones demonstrates proof of principle. Therefore, the screening method was applied in screening of two site-saturation libraries of Tat-PAMO. In these libraries, the positions I67 or M446 were randomised, and for both libraries, 91 randomly picked clones were assayed. The libraries were tested for activity with benzyl phenyl sulfide, which is poorly accepted by wild-type PAMO (Dudek et al., 2011). In the screening of the M446X library, a reaction time of 90 min was applied, and clones producing at least 1.4 mM phosphate were considered as active. To minimise false positive hits, we excluded clones displaying the fluorescence of
at least 80% of the Q152F/L153G/M446G mutant. Using these threshold values, eight active clones were isolated from the M446X library. Out of these hits, the previously characterised M446G variant was found seven times, while also a M446A mutant was found. To analyse the properties of the newly found M446A mutant in more detail, both enzymes were isolated and their reactivity towards benzyl phenyl sulfide was tested. The M446A mutant proved to convert this substrate with higher efficiency than the M446G variant (70% conversion in the case of M446A and 59% conversion in the case of M446G) and similar excellent enantioselectivity, leading to the (R)-sulfoxide as the main product (in both cases more than 98% ee). The I67X library was screened using less stringent threshold values, since initial experiments showed overall lower activity among mutants in this library. Therefore, a reaction time of 3 h was applied, and clones producing at least 0.8 mm phosphate were further analysed. Under these conditions, eleven clones were isolated, and ten of these were found to be the I67S variant whereas I67T was obtained once. Both mutants were purified and their reactivity towards benzyl phenyl sulfide was studied. While the ability of I67T to convert benzyl phenyl sulfide was reported before (Dudek et al., 2011), the I67S mutant also produced the sulfoxide with high enantiomeric excess (98% ee for the (S)-enantiomer).

We also picked several clones displaying highest fluorescence (NADPH oxidase activity) in each library. It turned out that all four uncoupling clones selected from the I67X library were I67P mutants. Three uncoupling mutants from the M446X library contained a M446W mutation. These mutations, that is, the introduction of a bulky residue at position 446 or a proline instead of an isoleucine at position 67, are indeed expected to perturb the active-site architecture, resulting in uncoupling mutants, which, thereby, further confirms the accuracy and the reliability of our screening method.

Discussion

Directed evolution experiments often lead to the conclusion that the screening conditions should reflect as much as possible the real conditions under which the reaction is to be performed. Use of surrogate or labelled substrates may result in identification of mutants less active on the target compound or not active at all. Furthermore, activity assays are often based on the particular property of a substrate or a product and thus need to be established for each compound. This underscores the need for a generic screening procedure applicable to a given class of enzymes and which is compatible with unmodified substrates.

BVMOs can perform different types of oxidations: Baeyer–Villiger oxidation, sulfoxidation, epoxidation, and oxidation of heteroatoms like nitrogen, boron, and selenium (Balke et al., 2012; Leisch et al., 2011). A truly generic method for this
class of enzymes would enable screening for all these different activities. Therefore, a generic method can be related to oxygen or NADPH consumption by BVMOs during catalysis. However, accurate measurements of oxygen depletion would be difficult to perform in a high-throughput manner because oxygen diffuses easily, and a tight closure of the reaction system would be required. Moreover, oxygen is consumed by cellular metabolism, which would interfere with a whole-cell activity screening method based on oxygen consumption. On the other hand, following NADPH depletion is a routinely used activity assay for BVMOs. In order to apply this method for screening libraries of mutants, cell extracts need to be prepared first because there are no transporters for NADPH in the cell membrane. Lysing cells is a time- and labour-consuming step. Moreover, cellular NADPH-dependent enzymes are released, which interferes with the measurement, and high expression levels or high activities of the targeted enzyme are necessary to reliably detect its activity in the cell extract. Lastly, the absorbance spectrum of NADPH overlaps with spectra of many potential substrates, which would hamper accurate determination of NADPH.

Here we have addressed the above-mentioned issues and have developed a generic, whole-cell-based procedure suitable for screening BVMOs in 96-well plate format. Our method revolves around periplasmic expression of the target enzyme in *E. coli* because periplasmic location offers unrestricted access of the substrate to the enzyme, as has been shown in previous studies (Sroga and Dordick, 2001). Additionally, directing the enzyme outside the cytoplasm helps to separate the activity of interest from cytoplasmic enzymes which can interfere with the activity assay. Based on the cofactor requirements of PAMO, we decided to use the Tat pathway for periplasmic transport. Our data indeed show that PAMO is transported to the periplasm in a functional form. Importantly, PAMO is the first flavoprotein monooxygenase expressed functionally in the periplasm.

Moreover, periplasmic expression of PAMO enables efficient coenzyme recycling by the established PTDH-based regeneration system (Torres Pazmiño et al., 2008; Torres Pazmiño et al., 2009), using externally added PTDH. We were able to detect and quantify the phosphate released by this system during NADPH regeneration in a colorimetric reaction with molybdate (Saheki et al., 1985), allowing the use of the released phosphate as a reporter of BVMO activity. In this way, we could accurately measure BVMO activity using whole-cells. The method allows end-point measurements and the detected phosphate amount corresponds to the amount of the oxidation product. Although it is possible to spectrophotometrically observe NADPH consumption by *E. coli* cells expressing PAMO in the periplasm, the accuracy is affected by light-scattering effects of cells. Here, by using indirect measurements of NADPH depletion, we can move the detection to the visible range and use high cell densities. Employing the regeneration system
further increases the sensitivity of the method because the amount of phosphate can build up during multiple turnovers. This principle could also be applied to cell extracts, but in this case, the background would be much higher because of cellular NADPH-consuming enzymes.

BVMOs are known to produce hydrogen peroxide in the absence of a suitable substrate while consuming NADPH. This form of non-productive catalysis is undesirable in our screening method because it increases the number of false positive hits. While in wild-type PAMO uncoupling occurs at a low rate (Torres Pazmiño et al., 2008), this reaction can be promoted by mutations introduced in the active site during creating libraries. To increase the accuracy of our screening method, we included an additional control reaction to monitor the formation of hydrogen peroxide. To this end, we successfully used Peroxy Green 1, a fluorescent dye highly specific for hydrogen peroxide (Miller et al., 2007). It is worth noting that our work represents the first example of using Peroxy Green 1 in mutant library screening.

We showed that the procedure enables distinguishing between active PAMO variants and inactive variants and that the amount of phosphate produced during biotransformation is in agreement with the amount of the oxidation product. This clearly demonstrated proof of principle, thereby validating our method and confirming its robustness. Subsequently, two site-saturation libraries of Tat-PAMO, randomised at positions I67 or M446, were successfully screened using our protocol, resulting in the identification of two novel PAMO variants with interesting enantioselectivities. Notably, screening of other libraries using this procedure is currently on-going in our laboratory.

Our newly developed method offers the advantage of screening for the activity towards unmodified substrates. In fact, the same protocol can be employed for any potential substrate. In this report, we have used the method in screening for activity on phenylacetone and benzyl phenyl sulfide. These substrates undergo different reaction types, namely the Baeyer–Villiger oxidation and sulfoxidation. Since the enzyme is expressed in the periplasm, which is a weakly controlled cellular compartment, it is possible to screen for activity or stability in different reaction media (for instance, at pH different than neutral or in the presence of solvents). This points out to the flexibility of the protocol. Moreover, the applications of the new method are much broader than just screening for BVMO activity. In principle, the method can be applied in the screening of any NADPH-consuming enzyme. Moreover, since PTDH accepts both NADPH and NADH, this method can be used to screen for NADH-dependent enzymes as well, or it could be employed to evolve enzymes with altered coenzyme specificity. Even though the method is based on the general ability of enzymes to oxidise NADPH, it allows immediate elimination of variants catalysing unproductive cycles, generating hydrogen
peroxide. The established screening protocol is suitable for 96-well plates which obviously limits the throughput to several hundred clones per day. We are currently exploring options to translate the reported principle of screening to a higher throughput format, for example, by growing and screening colonies on agar plates.

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