

Rapid Methods for High-Throughput Detection of Sulfoxides

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ABSTRACT

Enantiopure sulfoxides are prevalent in drugs and are useful chiral auxiliaries in organic synthesis. The biocatalytic enantioselective oxidation of prochiral sulfides is a direct and economical approach for the synthesis of optically pure sulfoxides. The selection of suitable biocatalysts requires rapid and reliable high throughput screening methods. Here we present four different methods for detecting sulfoxides that were produced *via* whole cell biocatalysis, three of which were exploited for high throughput screening. Fluorescence detection based on the acid activation of omeprazole was utilized for high throughput screening of mutant libraries of toluene monooxygenases, however no active variants were discovered yet. The second method is based on the reduction of sulfoxides to sulfides with the coupled release and measurement of iodine. The availability of solvent-resistant microtiter plates enabled to modify the method into high throughput format. The third method, selective inhibition of horse liver alcohol dehydrogenase, was used to rapidly screen highly active and/or enantioselective variants towards (*S*)-methyl-*p*-tolyl sulfoxide in a saturation mutagenesis library at position V106 of toluene *ortho*-monooxygenase. A success rate of 89% (i.e. 11% false positive) was obtained and two new mutants were selected. The fourth method is based on the colorimetric detection of adrenochrome, a back-titration procedure which measures the concentration the periodate-sensitive sulfide. Due to low sensitivity during whole-cell screening, this method was found to be useful only for determining the presence or absence of sulfoxide in the reaction. The methods described in the present work are simple, inexpensive and do not require special equipment.

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INTRODUCTION

The growing demand for green catalytic processes has increased utilization of enzymes as industrial biocatalysts for the synthesis of fine chemicals (6, 20, 21). As a consequence, there is a continuous search for novel or improved biocatalysts. In order to find an appropriate candidate for a process, various sources of enzymes must be screened for activity (24). Therefore, a sensitive, reproducible, accurate and simple high throughput screening method is a key prerequisite for the development of biocatalytic processes on an industrial scale (18, 40).

Screening systems are divided into three different classes. The first class contains assays applicable to test growing or resting microbial colonies for enzymatic activity directly on agar plates (24). For example, detection of epoxide hydrolase activity on butane oxide using safranin O. Oxidation of the 1,2-diol product by *E. coli* modified the membrane potential and led to accumulation of the red dye in the colonies producing active enzyme (35). In another study, the spontaneous oxidation of substituted catechols to brown-red quinones, was used to screen random libraries of whole cells expressing toluene monooxygenases for regioselective oxidation of substituted phenols (12, 32). The positive clones produced a red halo around the cells. These type of assays are high throughput and simple, but often require a tailored substrate with a chromophore such as bromonaphthol or azo-dye (24).

The second class includes chromogenic and fluorogenic assays applicable in microtiter plate or microarray formats (24). Microtiter plates in 96 or 384 wells format are particularly well-suited for spectroscopic reading using either UV/VIS or fluorescence plate readers. This class may be sub-divided into four groups: (i) Enzyme coupled assays, such as the determination of dehydrogenase activity through formation of NADH from NAD and absorbance change at wavelength of 340 nm; (ii) Chromogenic and fluorogenic substrates, such as the various synthetic labeled substrates that are commercially available and for the determination of hydrolytic activity produced by lipases, phosphatases, glycosidases, amidases, etc.; (iii) Chromogenic and fluorogenic sensors such as widely-used pH-indicators (16) that may be applied in any reaction that includes a change in pH; and (iv) Microarrays using a solid support enable screening of thousands of samples. The high throughput potential of this method was

demonstrated by profiling 40 different esterases and lipases across 35 different fluorogenic ester substrates using only 50 μ l of each enzyme solution and sub milligram of each substrate for over 7,000 tests (2).

5 The third class of enzymatic assays rely on product detection by instruments such as gas chromatography (GC), high pressure liquid chromatography (HPLC), mass spectrometry, nuclear magnetic resonance (NMR) spectrometry or infrared radiation that have been adapted for high throughput (23, 24, 34). Such assays require expensive and sophisticated equipment, but they allow working directly with the substrate of interest and are rapidly adapted once the instrument is available (24).

10 Various chemical substances can be synthesized by bacteria and fungi, among them are the chiral sulfoxides (5, 10, 11, 25, 37). As natural products, chiral sulfoxides possess a wide range of biological activities from flavor and aroma precursors to antimicrobial properties. In addition, they are efficient auxiliaries that lead to essential asymmetric transformations (11, 26). Furthermore, one of the most significant applications of chiral sulfoxides is in the pharmaceutical industry (3). The world's highest selling anti-ulcer drug, (*S*)-omeprazole, is a chiral sulfoxide (11, 14). Although there have been numerous reports on chemical and biological methods for synthesizing chiral sulfoxides, little information exists about rapid high throughput assays for sulfoxide determination. In this study, four colorimetric or fluorometric procedures were evaluated and adapted for screening of whole cell libraries containing variants of toluene monooxygenases (TMOs). Three of the four methods were successfully exploited to high throughput format using 96-well microtiter plates, whereas one method was not suitable due to low sensitivity. The method based on acid activation of omeprazole proved very efficient but no positive variants were found, whereas the one based on selective inhibition of horse liver alcohol dehydrogenase (HLADH), originally reported by Sprout and Seto (30), was useful for detecting mutants with high activity and enantioselectivity in the oxidation of methyl *p*-tolyl sulfide.

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MATERIALS AND METHODS

- Chemicals.** Thioanisole (99%), methyl *p*-tolyl sulfide (99%), methyl phenyl sulfoxide (97%), (R)- and (S)-methyl *p*-tolyl sulfoxide (98%), liver acetone powder (originated from horse), β -NAD and adrenaline hydrochlorate were purchased from Sigma-Aldrich (Rehovot, Israel). Sodium *meta*-periodate and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Tri-fluoroacetic acid anhydride was purchased from Riedel-de Haën (Seezle, Germany). Sodium iodide was purchased from Spectrum (New Brunswick, NJ, USA). Omeprazole-sulfide was received from TAMI-IMI Institute for R&D (Haifa, Israel). (S)-omeprazole was obtained from Teva (Teva, Petach-Tikva, Israel). All materials used were of the highest purity available and were used without further purification.
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- 10 **Bacterial strains and growth conditions.** *Escherichia coli* TG1 (*supE hsd Δ 5 thi Δ (lac-proAB) F'* [*traD36 proAB⁺ lacI^f lacZ Δ M15*]) with the plasmid constructs was routinely cultivated at 37°C with shaking at 250 rpm on a TU-400 incubator shaker (Orbital shaker incubator, MRC, Holon, Israel) in Luria-Bertani (LB) medium (28) supplemented with kanamycin at 100 μ g/ml (LBK) to maintain the plasmids. To stably and constitutively express the toluene monooxygenase (TMO) genes from the same
- 15 promoter, the expression vectors pBS(Kan)TOM (7), pBS(Kan)TpMO (33), pBS(Kan)ToMO (36), and pBS(Kan)T4MO (33), were constructed as described earlier. Expression of TMOs (wild-type and protein variants) by pBS(Kan) vectors within *E. coli* strains produced blue- or brown-colored cells on agar plates and in broth cultures. The color is indicative of indigoid compounds formed by oxidation of indole from tryptophan (9, 27).
- 20 **Screening methods. (i) Fluorescence test for omeprazole.** Screening for mutants with improved activity was performed by picking colonies from glycerol stocks stored in 96-well plates using a library copier VP 381 (V&P scientific, Inc., San Diego, CA, USA) and transferring into 96 deep well plates with a plastic lid (ABgene, Thermo Fisher Scientific, Epsom, United Kingdom) containing 1.2 ml of LBK. The cells were grown for 18 h at 37°C with shaking at 250 rpm in a TU-400 incubator shaker, followed by
- 25 centrifugation at 3,000 x g for 10 min, at 25°C using a Sigma-4K15 centrifuge (Sigma, Osterode, Germany). The cell pellets were re-suspended in 350 μ l phosphate buffer (PB) 20 mM, pH 7, from which

100 μ l were removed to measure the cell density (OD at 600 nm, approximately 1.1). The substrate omeprazole sulfide was added to 250 μ l of bacterial suspension to a final concentration of 0.25 mM (from a 12.5 mM stock solution in ethanol), and the biotransformation was carried out for 48 h with shaking at 600 rpm using a Vibramax 100 shaker (Heidolph, Nurenberg, Germany) at 25°C. The reaction was
5 stopped by additional centrifugation at 3,000 x g for 10 min, at 25°C. 100 μ l of the supernatant were transferred to F96 MicroWell™ black plates (Nunc, Roskilde, Denmark). 30 μ l of HCl 0.1N, were added to each well. The plate was incubated for 5 min at room temperature and the fluorescence signal was measured (emission 370 nm, excitation 560 nm) using a multiplate reader Synergy HT (BioTek Instruments, VT, USA). All liquid handling steps were conducted using an *epMotion 5070* robotic system
10 (Eppendorf AG, Hamburg, Germany).

(ii) Adrenaline test. Omeprazole-sulfide, thioanisole and methyl-*p*-tolyl sulfide were examined as potential substrates in this test. The procedure included bacterial growth in 5 ml LBK for 20 h at 37°C with shaking at 250 rpm (TU-400 incubator shaker), followed by cell harvesting at 8,000 x g for 10 min, at 25°C (Sigma-4K15 centrifuge). The cell pellets were re-suspended in 2.5 ml PB 20 mM, pH 7, to a
15 final OD of 4. The substrates were added (0.5 mM substrate from a 50 mM stock solution in ethanol) to 1 ml of cell suspension and the biotransformation was carried out for 3 h in a 16 ml glass vial, with shaking at 600 rpm, using a Vibramax 100 shaker at 25°C. The reaction was stopped by centrifugation (13,400 x g for 1 min.) using a Minispin centrifuge (Eppendorph, Hamburg, Germany). 850 μ l of the supernatant were transferred to a new vial, which was then incubated with 50 μ l NaIO₄ (10 mM stock in water) for 20
20 min. Subsequently, adrenaline-hydrochloride 150 μ l (15 mM stock in water) was added and incubated for 5 min, followed by measurement of color at 490 nm (Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden). The total assay volume was 1050 μ l.

(iii) TFAA-NaI test. Bacterial growth was performed as described in section (i). The growth was followed by centrifugation at 3,000 x g for 10 min, at 25°C using a Sigma-4K15 centrifuge (Sigma,
25 Osterode, Germany). The cell pellets were re-suspended in 600 μ l phosphate buffer (PB) 0.1 M, pH 7,

from which 100 μ l were removed to measure the cell density (OD at 600 nm, \sim 1). The substrate, methyl phenyl sulfide, was added to 500 μ l of bacterial suspension to a final concentration of 1 mM (from a 50 mM stock solution in ethanol), and the biotransformation was carried out for 5 h with shaking at 750 rpm using a Vibramax 100 shaker (Heidolph, Nuremberg, Germany) at 25°C. The reaction was stopped by additional centrifugation at 3,000 x g for 10 min, at 25°C. 400 μ l of the supernatant were transferred to a new polypropylene deep well plate (ABgene, Thermo Fisher Scientific, Epsom, United Kingdom) and 400 μ l of ethyl acetate were added to each well (1:1 volume) for the extraction of reactants. Phase separation, after vigorous pipettation, was facilitated by an additional centrifugation step (3,000 x g for 10 min), and 100 μ l of the organic phase were transferred to a new polypropylene 96-well plate (Grenier®) with a flat bottom (Sigma-Aldrich, Rehovot, Israel). Trifluoroacetic acid anhydride (TFAA) (10 μ l from 0.5 M stock in acetone) and sodium iodide (NaI) (100 μ l from 0.5M stock in acetone) were added followed by vigorous pipettation and a 2 min incubation at room temperature. The total assay volume was 210 μ l. Determination of the iodine released in the reduction reaction was measured at 362 nm in a multi-plate reader (Molecular Devices, Sunny Vale, CA, USA).

15 **(iv) Selective inhibition of horse-liver alcohol dehydrogenase (HLADH).** Bacterial growth conditions were as described in section (i), utilizing the library copier VP 381 and the *epMotion 5070* liquid handling system. The cell pellets were re-suspended in 350 μ l sodium phosphate buffer 46 mM, pH 7, to a final OD of 1.1. The substrate methyl *p*-tolyl sulfide was added to 250 μ l of bacterial suspension (100 μ l of the suspension were utilized for OD measurement) to a final concentration of 1 mM (from a 50 mM stock solution in ethanol), and the biotransformation was carried out for 4h with shaking at 600 rpm using a Vibramax 100 shaker at 25°C. The reaction was stopped by additional centrifugation at 3,000 x g for 10 min, at 25°C using a Sigma-4K15 centrifuge. 90 μ l of supernatant from each well were transferred to a new 96-well polystyrene plate (Nunc, Roskilde, Denmark). 10 μ l of freshly prepared liver acetone powder solution (from 10 mg/ml filtered stock solution in 46 mM ice-cold sodium phosphate buffer, pH 7) were added. The reaction was initiated by adding 90 μ l of freshly prepared 100 mM ethanol and 30 mM β -

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NAD in 46 mM ice-cold sodium phosphate buffer, pH 7. The total assay volume was 190 μ l. Absorbance data at a wavelength of 340 nm was collected over a period of 180 min. using a multi-plate reader (Molecular Devices, Sunny Vale, CA, USA).

Validation methods. In order to confirm the accuracy and reproducibility of the screening methods, 5 examined variants were also analyzed using GC or HPLC analysis. The bacteria were grown as described in the screening method section, and after the biotransformation the supernatant was designated either to screening or to GC/HPLC analysis.

Analytical methods. Conversion of thioanisole and methyl *p*-tolyl sulfide to the corresponding sulfoxides was determined by gas chromatography with a GC 6890N (Agilent Technologies, Santa Clara, 10 California) using a 30 m \times 0.32 mm \times 0.25 μ m capillary column packed with γ -cyclodextrin trifluoroacetyl (CHIRALDEX™ G-TA, ASTEC, Bellefonte, Pennsylvania) and a flame ionization detector. The temperature for thioanisole was programmed as follows: T₁=110°C; dT/dt=10°C/min, T₂=130°C; dT/dt=20°C/min, T₃=160°C, 13 min, split ratio 1:3. Under these conditions, the retention times were 3.89 min for thioanisole, 10.35 min for (R)-methyl phenyl sulfoxide, and 14.15 min for (S)-methyl phenyl 15 sulfoxide. For determination of methyl *p*-tolyl sulfide conversion, the temperature was programmed as follows: T₁=110°C; dT/dt=10°C/min, T₂=130°C; dT/dt=20°C/min, T₃=160°C, 17.5 min, split ratio 1:3. Under these conditions, the retention times were 4.45 min for methyl *p*-tolyl-sulfide, 12.56 min for (R)-methyl *p*-tolyl sulfoxide, and 13.46 min for (S)-methyl *p*-tolyl sulfoxide.

For the oxidation of omeprazole-sulfide to esomeprazole, an HPLC method was developed. The 20 samples were diluted in H₂O/acetonitrile 75%/25%, and filtered using a PVDF 0.45 μ m filter (Millex HV, Millipore, Cork, Ireland). The mobile phase was set as 70/30 H₂O/acetonitrile for 0-1 min, gradient to 40/60 H₂O/acetonitrile at 6 min, and gradient to 70/30 H₂O/acetonitrile at 12 min, and the flow rate was 1 ml/min. Compounds were identified by comparison of retention times (omeprazole-sulfide - 7.1 min; esomeprazole - 4.8 min) and UV-visible spectra to those of authentic standards. Calibration curves were 25 made at the maximum wavelength of both compounds (300 nm).

Saturation mutagenesis. Saturation mutagenesis at position TmoA I100 in pBS(Kan)T4MO and

TomA3 V106 in pBS(Kan)TOM was described by Feingersch *et al.* (10). A gene library encoding all possible amino acids at position 285 of T4MO TmoA in pBS(Kan)T4MO was constructed by replacing the target codon with NNN (N stands for A, T, G, or C) *via* overlap-extension polymerase chain reaction (PCR). Two primers, BM_T4MO_285_Front and T4MO_285_Rear (Table 1) were designed to
5 randomize position 285 of TmoA. Two additional primers for cloning of each enzyme were T4MObefEcoRI Front and T4MOABRear (Table 1). Vent DNA polymerase (New England Biolab, Ipswich, MA, USA) was used in the PCR reaction to minimize random point mutations, and pBS(Kan)T4MO was used as the template. The first 1006 nucleotide degenerate fragment was amplified by PCR using two primers, T4MObefEcoRI Front and T4MO_285_Rear, and the second degenerate
10 fragment of 1062 nucleotides was amplified by PCR using two other primers, T4MO_285_Front and T4MOABRear. A PCR program consisted of an initial denaturation at 94°C for 2 min, 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2.2 min with final extension of 72°C for 8 min. The two fragments were combined at a 1:1 ratio as templates to obtain the full-length degenerate PCR product (2034 bp) using T4MObefEcoRI Front and T4MOABRear as primers. The PCR program was programmed as
15 follows: initial denaturation at 94°C for 2 min, 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 3.15 min with final extension of 72°C for 8 min. The resulting PCR product containing randomized nucleotides at TmoA position 285 was cloned into pBS(Kan)T4MO after double digestion with EcoRI and AatII, replacing the corresponding fragment in the original plasmid. The AatII site occurs naturally within the TmoC gene and the EcoRI site is upstream of TmoA in the multiple cloning site. The resulting
20 plasmid library was transformed into *E. coli* TG1 competent cells *via* electroporation using MicroPulser electroporator (Biorad, Hercules, CA, USA).

Random mutagenesis *via* error-prone PCR (epPCR). The TmoA and TmoB genes, α and γ hydroxylase subunits, respectively, and 20% of TmoC gene (1936 bp) in pBS(Kan)T4MO were amplified using epPCR. A 100 μ l reaction contained 67 mM Tris-HCl (pH 8.8 at 25°C), 16 mM (NH₄)₂SO₄, 0.01%
25 Tween-20, 5 mM MgCl₂, 0.35 mM MnCl₂, 1 M Betaine, 40 ng of template DNA pBS(Kan)T4MO, 0.2 mM dATP and dGTP, 1 mM dCTP and dTTP, 5U Taq DNA polymerase (Bioline, BioTaq, London, UK),

and 30 pmole of each primer (T4MObefEcoRI Front and T4MOABRear, Table 1). The T4MObefEcoRI Front primer is upstream of the EcoRI restriction site, located in the multiple cloning site, and T4MOABRear primer is downstream of the naturally occurring AatII site within the TmoC gene.

TomA3 and TomA4, α and γ hydroxylase subunits, respectively, in pBS(Kan)TOM (2128 bp) were amplified using epPCR with the same conditions, but with primers TOM_FRONT_ep_np and TOM_Rear_ep_np (Table 1). The TOM_FRONT_ep_np primer is located upstream of the BlnI restriction site, and TOM_Rear_ep_np primer is downstream of the MfeI restriction site. The PCR programs used for DNA amplifications consisted of initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 1 min, 59.3°C or 58.3°C, for T4MO or TOM, respectively, for 1min, and 72°C for 2.5 min with final extension of 72°C for 7 min. The resulting randomized PCR products were cloned into pBS(Kan)T4MO or pBS(Kan)TOM after double digestion with AatII and EcoRI or BlnI and MfeI (New England Biolabs, Beverly, MA, USA), respectively, replacing the corresponding fragment in the original plasmid. The resulting plasmid library was transformed into *E. coli* TG1 competent cells *via* electroporation using MicroPulser electroporator.

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RESULTS

(i) **Fluorescence assay for omeprazole.** The production of omeprazole from omeprazole-sulfide was based on the acid-activation of omeprazole, which results in the development of a fluorescent signal at an excitation wavelength of 370 nm and emission at 560 nm (31) (Figure 1a). Under the experimental conditions, omeprazole-sulfide remains inactivated and does not interfere with the fluorescence signal (Figure 2a). A calibration curve prepared in buffer using increasing concentrations of omeprazole, was linear with the fluorescence signal indicating that the presence of omeprazole in the reaction mixture could be detected using this method (Figure 2b). In order to establish optimal conditions for the assay, several parameters were examined, including sample volumes, assay medium (LBK or PB), ionic strength of the biotransformation buffer, and pH of sample with HCl (Figure 2a). Eventually, this technique was modified for high throughput screening of bacterial libraries in 96-well microtiter plates using the

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epMotion 5070 liquid handling system. The process involves bacterial growth and biotransformation in 96-deep well plates, transfer of the supernatant to F96 MicroWell™ black plates, addition of HCl followed by incubation for 5 min and fluorescence measurement using a multi plates reader. The use of LBK medium for the biotransformation resulted in a high fluorescence signal and interfered with the results (Figure 2a); therefore, the biotransformation was performed in buffer and the cells were removed prior to addition of HCl. Phosphate buffer at an ionic strength of 100 mM was inappropriate for the assay (results not shown), but 20 mM was adequate as depicted from the control sample (Figure 2a). The optimal pH for the reaction was determined to be 2.

Random libraries of T4MO and TOM were prepared using epPCR as well as three saturation mutagenesis libraries at positions TOM V106, T4MO I100 and D285. Residue V106 in TOM and the analogous position I100 in T4MO were shown in previous works to influence regio- and enantioselectivity, and were therefore a prime target here. For instance, T4MO I100A and I100S were highly active in the hydroxylation of nitrobenzene (12), whereas I100G oxidized methyl *para*-tolyl sulfide 11 times faster than the wild type and changed the selectivity from 41% pro-*R* to 77% pro-*S* (10). TOM V106A had increased activity on various chlorinated ethenes (7) as well as improved enantioselectivity on thioanisole (10). T4MO D285 is situated near the protein surface in the entrance to the active site channel and was chosen based on homology modeling studies (unpublished results). This previously-unstudied position is suggested to control substrate entrance and product efflux. Thousands of colonies from these libraries were screened using the high throughput method described; however, no active variants on omeprazole-sulfide were found to date.

An attempt was made to apply the fluorescence test of omeprazole for detection of additional sulfoxides, such as methyl phenyl sulfoxide and methyl *p*-tolyl sulfoxide; however, there was no difference between the signals of the sulfides and their corresponding sulfoxides. Thus, this assay is specific for omeprazole.

(ii) **Adrenaline test.** This method is a back-titration procedure which measures the concentration of periodate-sensitive substances (39). In the original publications, diols and epoxides were used as

substrates (38, 39). In the present TMO-catalyzed reaction, sulfoxide is produced using whole cells, and the remaining sulfide is oxidized with excess sodium periodate, followed by reaction of the remaining periodate with adrenaline to form the red dye adrenochrome (Figure 1b). It is expected that the more sulfoxide produced by the cells, the more intense the red color is (less sulfide remains, therefore more sodium periodate oxidizes adrenaline and more adrenochrome is created). Various parameters were evaluated during the development of the adrenaline test, including substrate concentration, incubation times with NaIO₄ and adrenaline-hydrochloride, ionic strength of PB pH 7.0, and concentration of NaIO₄. It was found that the reaction can not be performed in LBK medium, since no color appears, perhaps due to oxidation by sodium periodate of other substances present in the medium such as glycerol (13). Additionally, it was found that the ionic strength of the phosphate buffer influences the absorbance (the absorbance is higher when 20 mM phosphate buffer is used compared to 100 mM). Finally, it was discovered that presence of cells interferes with absorbance measurement; therefore, an additional centrifugation is needed to obtain cell-free medium (results not shown).

Three sulfides and their respective sulfoxides were examined using the adrenaline test: methyl phenyl sulfide and methyl phenyl sulfoxide, methyl-*p*-tolyl sulfide and (S)- and (R)-methyl-*p*-tolyl-sulfoxide, and omeprazole-sulfide and esomeprazole. Methyl phenyl sulfide and methyl *p*-tolyl sulfide and their corresponding sulfoxides showed good correlation between absorbance at 490 nm and increasing sulfoxide concentrations (Figure 3). But when the method was tested on wild type (WT) TMOs and their variants, the results did not correlate with those of the GC making this assay not sensitive enough for evaluation of highly active mutants. In addition, omeprazole showed no correlation between color intensity and sulfoxide concentration (Figure 3); hence, this method was found to be inappropriate as a high throughput screening exam.

(iii) TFAA-NaI test. This method is based on the use of trifluoroacetic anhydride-sodium iodide mixture as a reducing agent, converting sulfoxides to the corresponding sulfides, followed by the release of iodine whose yellow color is measured at 362 nm (17) (Figure 1c). There is a direct correlation between sulfoxide production and intensity of the yellow color. Originally, this method was developed for

determination of chemically-synthesized S-oxo and N-oxo compounds (17) and we modified it for determination of microbial sulfoxidation reactions. The bacterial oxidation of methyl phenyl sulfide to methyl phenyl sulfoxide was used as a model reaction. The original protocol used acetone as a medium however this solvent did not extract well the substrate and the product, and was found to damage 96-well polystyrene plates. A mixture of glacial acetic acid and acetic anhydride (95:5 v/v) was compatible with the plates, but it provided a very intense yellow color which limited absorbance reading without massive dilution of the samples. The liberated iodine could only be determined by an additional step of back titration with sodium thiosulfate, using starch as an indicator. The use of PB pH 7 as a reaction medium was examined at different ionic strengths (20 mM and 100 mM), but there was insufficient correlation between the color intensity and sulfoxide concentrations, and a long incubation time (80 min) was required. Therefore, it was concluded that an organic solvent is necessary for the TFAA-NaI reaction. Acetone was replaced with ethyl acetate due its superior extraction of methyl phenyl sulfide and methyl phenyl sulfoxide following the biotransformation. In addition, different concentrations and ratios of TFAA and NaI were examined (15-100 mM and 0.15-1 M, respectively). Eventually, the procedure consisted of 400 μ l ethyl acetate as an extractant, 20 mM TFAA and 0.2 M NaI followed by an incubation time of 2 min and measurement of the absorbance at 362 nm. A linear correlation between sulfoxide concentration and absorbance was obtained using the method developed (results not shown). Upon testing the method on whole cells expressing TMOs it was discovered that a second centrifugation step is necessary to remove the cells prior to contact with ethyl acetate, to prevent the extraction of interfering indigoid colors present in the cells. As the assay is performed in organic media, it cannot be executed in 96-well polystyrene plates. Recently, solvent-resistant 96-well plates (made from polypropylene) were marketed by Sigma-Aldrich, enabling to exploit this simple and sensitive method to high throughput format. The final version of the assay, showed high correlation with GC measurements performed in parallel (Figure 4) indicating that the tested variants are more active than WT TOM.

25 **(iv) Selective inhibition of HLADH** This assay is a modification of the method described by Sprout and Seto (30), and is based on the different inhibition constants of (S)- and (R)-methyl-*p*-tolyl sulfoxide

on the oxidation of ethanol to acetaldehyde catalyzed by HLADH. The compounds act as uncompetitive inhibitors, with the (S)-enantiomer being a better inhibitor than the (R)-enantiomer. The signal is created by the accompanying hydrogenation of β -NAD to β -NADH, and is measured at 340 nm (Figure 1d). Thus, as (S)-methyl-*p*-tolyl sulfoxide concentrations increase, absorption from β -NADH decreases.

5 Following a few preliminary trials, it was discovered that HLADH is not available commercially anymore. An alcohol dehydrogenase from Baker's yeast was evaluated, but was not inhibited by methyl *p*-tolyl sulfoxide, probably due to different protein structure compared to the horse enzyme. Finally, crude horse liver acetone powder (LAP) was used successfully in the assay. Various parameters were examined, such as pH of the buffer solution (pH 7 or 8.8), influence of the presence of sulfide in the reaction,

10 volume and concentration of LAP, background provided by other substances in the solution, different ratios of β -NAD and ethanol, various substrate concentrations and an appropriate duration of the biotransformation and of the enzymatic reaction. It was found that the reaction can be performed in sodium PB pH 7, which is more suitable for bacterial activity. In addition, it was found that the optimal biotransformation time is 4h. During this time, product is already formed and the evaporation of the

15 substrate from the 96-well plates is negligible. It was also discovered that an appropriate time for the colorimetric test is 3 h, because during this time period the enzyme reaches its maximum inhibition and the color does not change significantly afterwards. It should also be mentioned that the LAP solution should be filtered prior to usage.

The applicability of the assay was evaluated using a saturation mutagenesis library of TOM V106 which has been previously screened in our lab on methyl phenyl sulfide (10). The five best mutants in that

20 study were also evaluated on methyl *p*-tolyl sulfide, so the results could be compared with the present screen. Initially, TOM WT, its variants and TG1/pBS(Kan) (the host without the enzyme insert was used as negative control) were analyzed using the HLADH-inhibition based assay. In parallel, each sample was injected to the GC (Fig. 5). The negative control showed the highest absorbance signal since no sulfoxide

25 was produced. Mutants with higher activity and/or selectivity than WT TOM towards the (S)-sulfoxide, exhibited low absorbance due to strong inhibition of the HLADH. Good correlation between the two

methods was obtained. Afterwards, a total of 700 variants from the TOM V106 library were screened twice on methyl-*p*-tolyl sulfide. In the second trial, the plates were inverted in order to neutralize the effect of the variant's location in the plate. After summarizing both screenings, the four best performing and one worst performing mutants were picked from each 96-well plate and analyzed using GC, making a
5 total of 35 variants (best performing = the lowest absorption at 340 nm; worst performing = the highest absorption at 340 nm). Among the 7 worst performing variants chosen, five were indeed inactive and two were false negatives. Samples referred to as false negative are variants that were active according to GC injections, but showed unexpected high absorbance at 340 nm in the screening assay. Among the 28 best performing variants chosen, 24 were more enantioselective compared to the WT and 25 were more active.
10 Three variants were false positives meaning they were inactive according GC injections but showed low absorbance at 340 nm. These results represent a success rate of 89% for this assay. Six highly active and six highly enantioselective variants (according to GC analysis) were sequenced and the results were compared to our previous results (10). The HLADH-inhibition assay led to the selection of one of the most active variants, V106S, and one of the most enantioselective variants, V106A found in our previous
15 study (10). Two new variants were discovered (V106N and V106K), since previous screening was performed on methyl phenyl sulfide and not on methyl *p*-tolyl sulfide. According to our results, the use of enzyme inhibition enables to select better variants, either more enantioselective, or more active, or both.

DISCUSSION

20 Chiral sulfoxides play a significant role in pharmaceuticals and in organic synthesis as functionalized substances and as important chiral auxiliaries (11, 15, 26). In addition, there is a growing demand for green catalytic processes (20, 29), therefore there is a continuous search for novel and better performing biocatalysts. Development of an efficient high throughput screening assay for determination of sulfoxide production is crucial for the discovery of new or improved biocatalysts that are able to
25 produce a wide spectrum of significant substances (22, 24). Various high throughput screening systems are known in the art, but most of them are suitable for hydrolytic reactions and for isolated enzymes or

chemically synthesized substances (24). A suitable method for measuring sulfoxide formation or enantioselectivity was not published yet according to our knowledge.

An appropriate screen should be able to detect and quantify sulfoxides in the presence of sulfides and be modified to a 96-well format for rapid measurement. The present study describes four different assays for determination of sulfoxides produced by whole cell biocatalysis: fluorescence test for omeprazole, the adrenaline test, TFAA-NaI test, and HLADH-inhibition for methyl *p*-tolyl sulfoxide determination. The first three methods determine the production of sulfoxides, and the fourth method measures the enantiomeric excess as well as the amount of the sulfoxide produced. Three of the methods, the fluorescence test for omeprazole, the TFAA-NaI test and HLADH-inhibition, were exploited for high throughput screening of TMO libraries and showed good correlation with HPLC or GC results, respectively.

It was previously reported by Morii *et al.* that acid activation of omeprazole provides a strong fluorescent signal (19). This property was used for the development of a high throughput screen for determining omeprazole production. During the optimization of the assay it was discovered that the fluorescence signal is influenced by the ionic strength of the phosphate buffer. A high buffer concentration resulted in a strong background signal which masked the desired sulfoxide signal. Additionally, the use of LBK as the reaction medium was examined in an attempt to reduce the work up steps, but the fluorescence signal was again high and caused a disturbing background.

The final screening procedure enabled analysis of several 96 well plates in parallel and once the acid was added to the plates, the reaction lasted for only 5 min.

Zhang *et al.* developed a statistical parameter, the Z'-factor, for evaluating high throughput assays (40). The Z'-factor calculated from the positive and negative controls, is a characteristic parameter for the quality of the assay itself without the intervention of the test compounds. If the Z'-value is small (negative or close to zero), it usually indicates that the assay conditions have not been optimized or that the assay format is not feasible for generating useful data. A value close to 1 implies an excellent assay. The Z'-factor for the fluorescence assay for omeprazole was calculated to be 0.89, indicating an excellent high

throughput assay (40). According to our knowledge, there are no reports on successful biocatalytic oxidation of omeprazole sulfide to (*S*)-omeprazole. Although the screening of thousands of TMO variants did not result in positive hits capable of generating (*S*)-omperazole, this assay may be of use for screening other potential oxidizing enzymes.

5 The adrenaline test for enzyme catalysis which was developed by Reymond and co-workers (39) is a versatile high throughput assay used for detection 1,2-diols, 1,2-aminoalcohols and α -hydroxyketones by colorimetry. One of its limitations is the fact that periodate reacts with all diol molecules that might be present in the medium, even the undesirable ones (13). Subsequently, the reaction must be performed in buffer and any traces of LBK should be removed. In addition, the ionic strength of the buffer should be
10 kept low in order to obtain higher absorbance signals. The present work describes an attempt to extend the range of this assay for the determination of sulfoxides by measurement of the remaining sulfide. The assay was found to be suitable for detection of methyl phenyl sulfoxide and methyl *p*-tolyl sulfoxide but not omeprazole. However, the sensitivity was not pronounced enough to allow differentiation between WT TOM and its variants, probably due to other substances that were secreted by the cells during
15 biotransformation and oxidized by sodium periodate. The *Z'*-factor calculated for the adrenaline test is equal to 0.18, which indicates low sensitivity. Therefore, it can be used only for determination of the presence or absence of sulfoxides but not to quantify combinations of sulfides-sulfoxides.

 The TFAA-NaI assay was developed initially for the analytical determination of compounds bearing a semipolar *X-O* bond including a variety of sulfoxides and sulfimides (17). It exploits inexpensive
20 and commercially-available reagents and provides visible color change within a short time. This method was found to be very accurate, reproducible and suitable for detection of various sulfoxides. In addition, the *Z'*-factor calculated for this test is equal to 0.52, which indicates good sensitivity. The availability of polypropylene 96-well plates enabled this method to be adapted successfully for high throughput format. This method is currently being evaluated in our lab for other pro-chiral sulfides.

25 The use of enzyme inhibition to measure the enantiomeric excess of a chiral sulfoxide was initially proposed by Sprout and Seto (30) and was exploited by us as a high throughput screening exam for whole

cell biocatalysis of methyl *p*-tolyl sulfoxide. In order to demonstrate the high throughput nature of the method, about 400 variants were tested in parallel over 3 hours. Similarly to other fluorogenic and chromogenic methods for determination of enantioselective substances such as *p*-nitrophenol chiral esters, the HLADH-inhibition test may be considered as a high throughput assay (22). Moreover, the calculated
5 Z'-factor of 0.82, indicates the high quality of this assay. In addition to its simplicity, this assay is versatile in nature, since alcohol dehydrogenases from different origins have different specificities toward a wide range of sulfoxides, including chiral sulfoxides (8). For example, when using (*R*)- and (*S*)-methyl phenyl sulfoxides as inhibitors, the inhibition constants (K_i) of alcohol dehydrogenase from equine liver are 270 and 33 μM , respectively, while the K_i values of *Hs- β 1* class 1 alcohol dehydrogenase from human are
10 1100 and 1800 μM , respectively (8). In this work it was also shown that the purity of the test-enzyme is not crucial as both pure HLADH as well as crude liver acetone powder worked well. Although only a small library of 700 variants was evaluated by this assay, it is expected that 10,000 colonies may be screened in 2-3 weeks, similarly to the colorimetric method described by Bottcher and Bornscheuer for whole cells expressing esterases (4).

15 The only drawback of the HLADH-inhibition method is the difficulty to distinguish between high enantioselectivity (e.g. V106M in Figure 5) and high conversion rate (e.g. V106L in Figure 5), since both result in low absorption measurements (1). Nevertheless, an industrial biocatalyst must be highly active and enantioselective, and these two properties are important to detect in a library. This method is currently being used in our laboratory for evaluation of more random libraries.

20 Although chromatography and NMR techniques have been developed in recent years for high throughput screening (23, 24), they require expensive equipment, and data analysis is time consuming. Methods shown in the present work are simple, inexpensive and do not require special equipment besides a multi plate reader.

25

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REFERENCES

1. **Abato, P., and C. T. Seto.** 2001. EMDee: An enzymatic method for determining enantiomeric excess. *J. Am. Chem. Soc.* **123**:9206-9207.
2. **Babiak, P., and J. L. Reymond.** 2005. A high-throughput, low-volume enzyme assay on solid support. *Anal. Chem.* **77**:373-377.
3. **Bentley, R.** 2005. Role of sulfur chirality in the chemical processes of biology. *Chem. Soc. Rev.* **34**:609-624.
4. **Bottcher, D., and U. T. Bornscheuer.** 2006. High-throughput screening of activity and enantioselectivity of esterases. *Nature Protocols* **1**:2340-2343.
5. **Boyd, D. R., N. D. S. Sharma, S. A. Haughey, M. A. Kennedy, B. T. McMurray, G. N. Sheldrake, C. C. R. Allen, H. Dalton, and K. Sproule.** 1998. Toluene and naphthalene dioxygenase-catalysed sulfoxidation of alkyl aryl sulfides. *J. Chem. Soc., Perkin Trans.I* **12**:1929-1933.
6. **Breuer, M., K. Ditrich, T. Habicher, B. Hauer, M. Kessler, R. Sturmer, and T. Zelinski.** 2004. Industrial methods for the production of optically active intermediates. *Angew. Chem. Int. Ed. Engl.* **43**:788-824.
7. **Canada, K. A., S. Iwashita, H. Shim, and T. K. Wood.** 2002. Directed evolution of toluene *ortho*-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J. Bacteriol.* **184**:344-349.
8. **Cho, H., and B. V. Plapp.** 1998. Specificity of alcohol dehydrogenases for sulfoxides. *Biochemistry* **37**:4482-4489.
9. **Eaton, R. W., and P. J. Chapman.** 1995. Formation of indigo and related compounds from indolecarboxylic acids by aromatic acid-degrading bacteria: Chromogenic reactions for cloning genes encoding dioxygenases that act on aromatic acids. *J. Bacteriol.* **177**:6983-6988.
10. **Feingersch, R., J. Shainsky, T. K. Wood, and A. Fishman.** 2008. Protein engineering of toluene monooxygenases for synthesizing chiral sulfoxides. *Appl. Environ. Microbiol.* **74**:1555-1566.
11. **Fernandez, I., and N. Khir.** 2003. Recent developments in the synthesis and utilization of chiral sulfoxides. *Chem. Rev.* **103**:3651-3705.
12. **Fishman, A., Y. Tao, W. E. Bentley, and T. K. Wood.** 2004. Protein engineering of toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 for synthesizing 4-nitrocatechol from nitrobenzene. *Biotechnol. Bioeng.* **87**:779-790.
13. **Goddard, J.-P., and J.-L. Reymond.** 2004. Recent advances in enzyme assays. *Trends Biotechnol.* **22**:363-370.
14. **Grimley, J.** 2006. Pharma Challenged: Market shifts and generic competition create dynamic environment for drug developers. *Chem. Eng. News* **84**:17-28.
15. **Holland, H. L.** 2001. Biotransformation of organic sulfides. *Nat. Prod. Rep.* **18**:171-181.
16. **Janes, L. E., C. A. Lowendahl, and R. J. Kazlauskas.** 1998. Quantitative screening of hydrolase libraries using pH indicators: Identifying active and enantioselective hydrolases. *Chem. Eur. J.* **4**:2324-2331.
17. **Kudzin, Z., M. Kudzin, J. Drabowicz, and A. Kotynski.** 2007. Trifluoroacetic anhydride-sodium iodide reagent. *Nature and applications. ARKIVOC* **vi**:112-171.
18. **Lan Tee, K., and U. Schwaneberg.** 2007. Directed evolution of oxygenases: Screening systems, success stories and challenges. *Comb. Chem. High Throughput Screen.* **10**:197-217.
19. **Morii, M., H. Takata, and N. Takeguchi.** 1989. Acid activation of omeprazole in isolated gastric vesicles, oxyntic cells, and gastric glands. *Gastroenterology* **96**:1453-1461.
20. **Poliakoff, M., and P. Licence.** 2007. Sustainable technology: Green chemistry. *Nature* **450**:810-812.
21. **Pollard, D. J., and J. M. Woodley.** 2007. Biocatalysis for pharmaceutical intermediates: The future is now. *Trends Biotechnol.* **25**:66-73.

22. **Reetz, M. T.** 2002. New methods for high-throughput screening of enantioselective catalysts and biocatalysts. *Angew. Chem. Int. Ed. Engl.* **41**:1135-1338.
23. **Reetz, M. T., A. Eipper, P. Tielmann, and R. Mynott.** 2002. A practical NMR-based high-throughput assay for screening enantioselective catalysts and biocatalysts. *Adv. Synth. Catal.* **344**:1008-1016.
24. **Reymond, J.-L., and P. Babiak.** 2007. Screening systems. *Adv. Biochem. Eng. Biotechnol.* **105**:31-58.
25. **Ricci, L. C., J. V. Comasseto, L. H. Andrade, M. Capelari, Q. B. Cass, and A. L. M. Porto.** 2005. Biotransformations of aryl alkyl sulfides by whole cells of white-rot *Basidiomyces*. *Enzyme Microb. Technol.* **36**:937-946.
26. **Ronald, B.** 2005. Role of sulfur chirality in the chemical processes of biology. *Chem. Soc. Rev.* **34**:609-624.
27. **Rui, L., K. F. Reardon, and T. K. Wood.** 2005. Protein engineering of toluene *ortho*-monooxygenase of *Burkholderia cepacia* G4 for regiospecific hydroxylation of indole to form various indigoid compounds. *Appl. Microbiol. Biotechnol.* **66**:422-429.
28. **Sambrook, J., and D. W. Russell.** 2001. *Molecular cloning: A laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, New York.
29. **Schoemaker, H. E., D. Mink, and M. G. Wubbolts.** 2003. Dispelling the myths--biocatalysis in industrial synthesis. *Science* **299**:1694-1697.
30. **Sprout, C. M., and C. T. Seto.** 2005. Using enzyme inhibition as a high throughput method to measure the enantiomeric excess of a chiral sulfoxide. *Org. Lett.* **7**:5099-5102.
31. **Takeguchi, N., T. Yamanouchi, H. Sakai, and M. Morii.** 1992. New fluorescent probes E3810 and methoxy E3810 for determining distributions of the apical membrane and the acidic compartment of gastric acid secreting cells. *Jpn. J. Physiol.* **42**:75-88.
32. **Tao, Y., A. Fishman, W. E. Bentley, and T. K. Wood.** 2004. Altering toluene 4-monooxygenase by active-site engineering for the synthesis of 3-methoxycatechol, methoxyhydroquinone, and methylhydroquinone. *J. Bacteriol.* **186**:4705-4713.
33. **Tao, Y., A. Fishman, W. E. Bentley, and T. K. Wood.** 2004. Oxidation of benzene to phenol, catechol, and 1,2,3-trihydroxybenzene by toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 and toluene 3-monooxygenase of *Ralstonia pickettii* PKO1. *Appl. Environ. Microbiol.* **70**:3814-3820.
34. **Trapp, O.** 2008. Gas chromatographic high-throughput screening techniques in catalysis. *J. Chromatogr. A* **1184**:160-190.
35. **van Loo, B., J. H. L. Spelberg, J. Kingma, T. Sonke, M. G. Wubbolts, and D. B. Janssen.** 2004. Directed evolution of epoxide hydrolase from *Agrobacterium radiobacter* toward higher enantioselectivity by error-prone PCR and DNA shuffling. *Chem. Biol.* **11**:981-990.
36. **Vardar, G., K. Ryu, and T. K. Wood.** 2005. Protein engineering of toluene-*o*-xylene monooxygenase from *Pseudomonas stutzeri* OX1 for oxidizing nitrobenzene to 3-nitrocatechol, 4-nitrocatechol, and nitrohydroquinone. *J. Biotechnol.* **115**:145-156.
37. **Vargas, R. R., E. J. H. Bechara, L. Marzorati, and B. Wladislaw.** 1999. Asymmetric sulfoxidation of a β -carbonyl sulfide series by chloroperoxidase. *Tetrahedron: Asymmetry* **10**:3219-3227.
38. **Wahler, D., O. Boujard, F. Lefevre, and J.-L. Reymond.** 2004. Adrenaline profiling of lipases and esterases with 1,2-diol and carbohydrate acetates. *Tetrahedron* **60**:703-710.
39. **Wahler, D., and J.-L. Reymond.** 2002. The adrenaline test for enzymes. *Angew. Chem. Int. Ed. Engl.* **41**:1229-1232.
40. **Zhang, J.-H., T. D. Y. Chung, and K. R. Oldenburg.** 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**:67-73.

Table 1. Primers used for random mutagenesis *via* epPCR and for sequencing *tmoA* and *tmoB* in TG1/pBS(Kan)T4MO and *tomA3* and *tomA4* in TG1/pBS(Kan)TOM and for saturation mutagenesis at position D285 in the *tmoA* gene in TG1/pBS(Kan)T4MO.

Primer	Nucleotide sequence
<u>Mutagenesis primers</u>	
T4MObefEcoRI Front	5'-CCATGATTACGCCAAGCGCG-3'
T4MOABRear	5'-TCCATGCTCTTCACTGTTGAC-3'
BM_T4MO_285_Front	5'-GGATTACTACACGCCGTTGGAGNNNCGCAGCCAG-3'
T4MO_285_Rear	5'-AACTCCTTGAATGACTGGCTGCGNNNCTCCAACGG-3'
TOM_FRONT_ep_np	5'-CTCCAGCAAATCCACATCAACC-3'
TOM_REAR_ep_np	5'-GGGGCTGAATGTTGAGTTGATAGG-3'
<u>Sequencing primers</u>	
T4MObefEcoRI Front	5'-CCATGATTACGCCAAGCGCG-3'
T4MO Seq1	5'-CCCGCATGAATACTGTAAGAAGGATCGC-3'
T4MO Seq2	5'-GCTCGTTGATAGATCTGGGCTTGGAC-3'
T4MO Seq3	5'-AATCTATTGAAGAGATGGGCAAAGACGC-3'
TOM_FRONT_ep_np	5'-CTCCAGCAAATCCACATCAACC-3'
TOM_BSIWI_FRONT (TOM1)	5'-CCGATGGAGAAAGTGTTCCTCGTACGAC-3'
TOM Seq 3	5'-CGCCACATGACACTCGGCATC-3'
TOM seq 4	5'-CGGCGACAAGTATCACTTCTGC-3'

FIGURE LEGENDS

- Figure 1.** Chemical reactions used in this study to detect sulfoxides. (a) Fluorescence-based assay for direct measurement of the omeprazole-HCl complex; (b) The adrenaline-based assay for measuring the remaining sulfide in the reaction (adapted from (39)); (c) TFAA-NaI reagent for the reduction of the sulfoxide and liberation of I₂; (d) Selective inhibition of horse liver acetone powder by methyl *p*-tolyl sulfoxide (adapted from (30)).
- Figure 2.** Fluorescence-based assay for direct measurement of HCl-activated omeprazole. (a) Fluorescence signal of different treated samples of omeprazole and omeprazole-sulfide (total concentration of 0.5 mM). Samples including cells and standards in LBK or 20 mM PB, were centrifuged and the supernatant was examined for fluorescence after activation with 1N HCl. (b) A calibration curve of omeprazole in 20 mM PB, as obtained from the multi-plate reader at excitation wavelength 370 nm and emission wavelength of 560 nm. Every bar in the graph includes at least three independent experiments (total concentration of substrate and product was 0.5 mM);
- Figure 3.** The adrenaline-based assay for measurement of the remaining sulfide in the biotransformation reaction. A calibration curve in 20 mM PB (without the presence of cells) is presented for methyl phenyl sulfoxide, methyl *p*-tolyl sulfoxide and omeprazole at wavelength 490 nm. Every bar in the graph includes at least three independent experiments (total concentration of substrate and product was 1mM).
- Figure 4.** Correlation of the TFAA-NaI test with GC measurements. TOM and V106 variants, as well as TG1 cells were examined by the two methods using 1mM methyl phenyl sulfide as a substrate. (a) GC measurements and (b) TFAA-NaI colorimetric measurements. Readings are normalized to cell density. Results represent an average of duplicates.
- Figure 5.** Correlation of the selective inhibition of HLADH test with GC measurements. TG1, TOM and few V106 variants were examined by two methods using 1mM methyl *p*-tolyl sulfide as a substrate. (a) GC measurements and (b) selective inhibition of HLADH test. Results represent an average of duplicates.

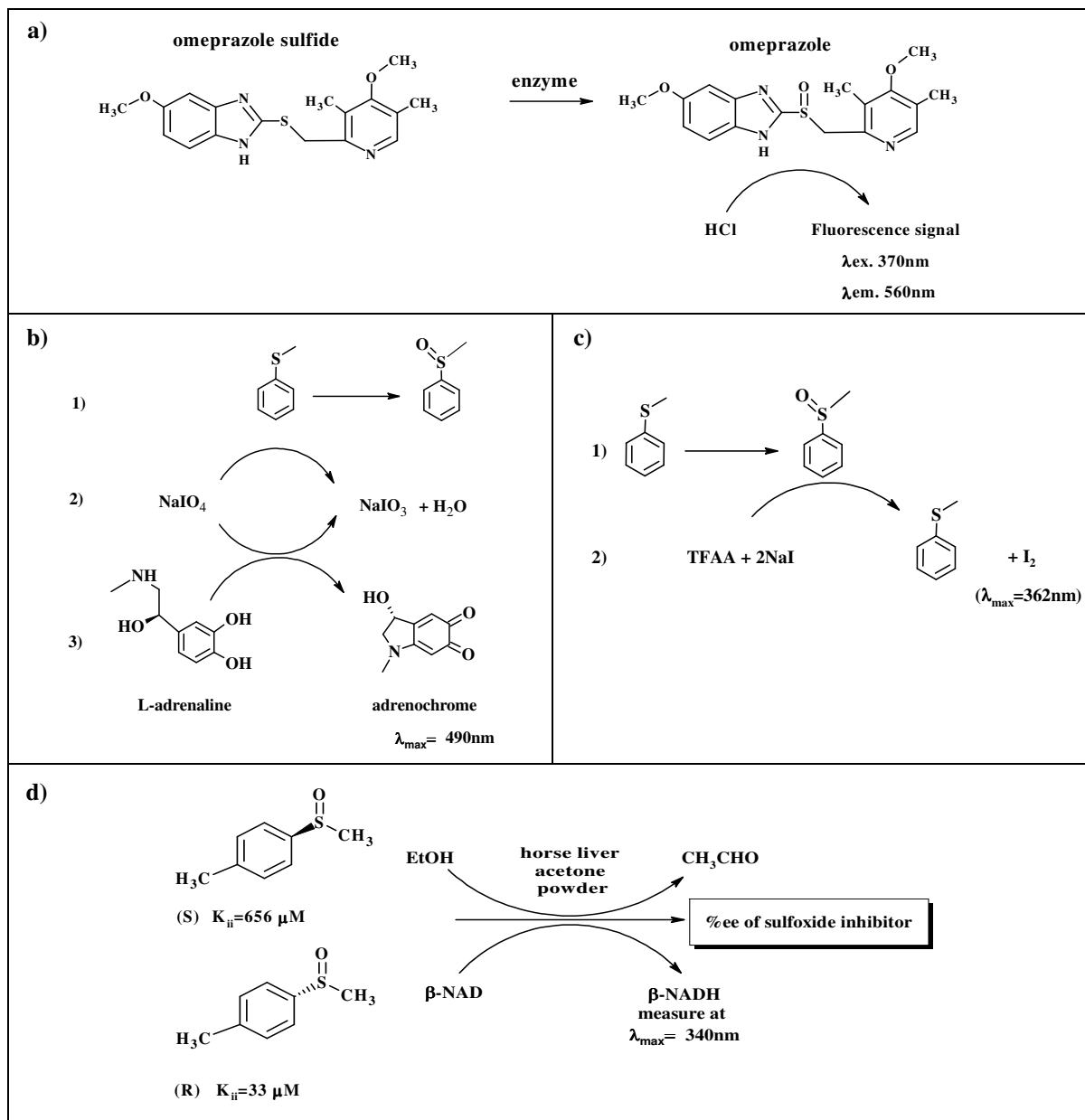


Figure 1

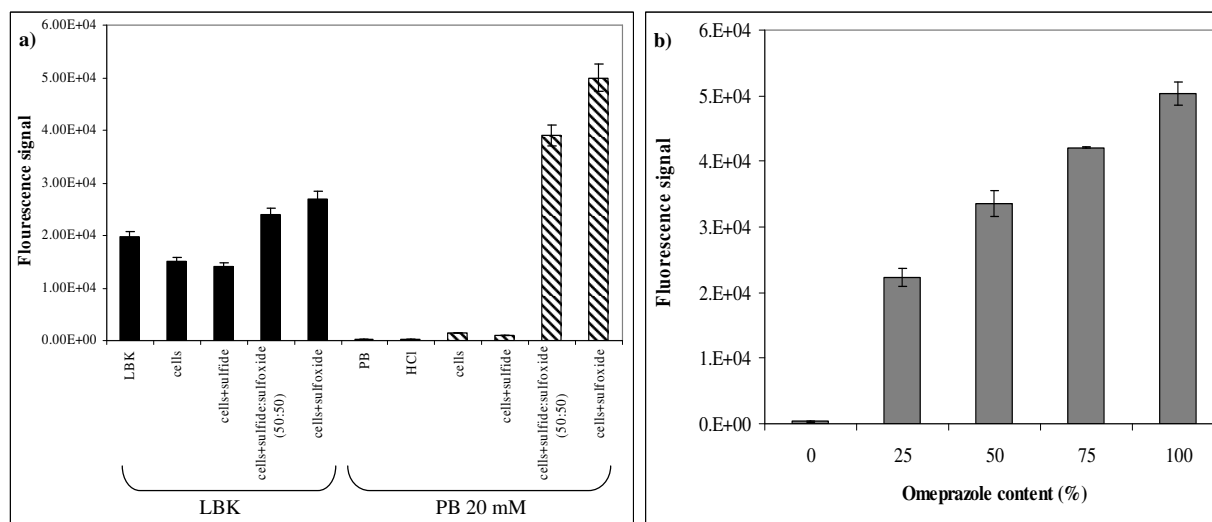


Figure 2

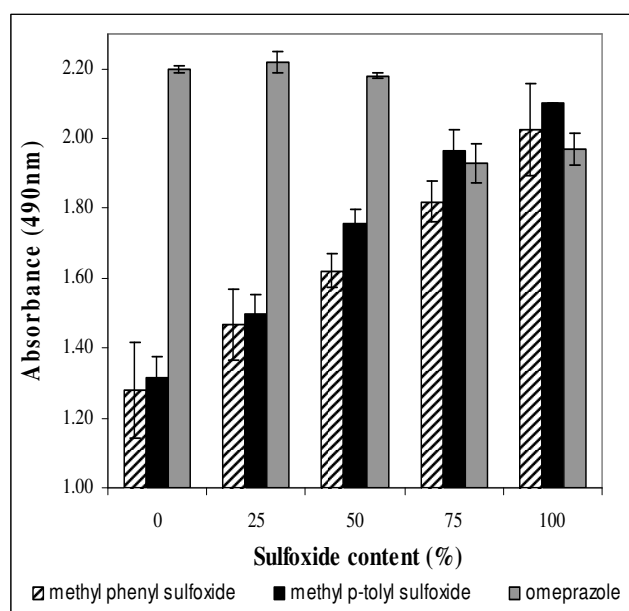


Figure 3

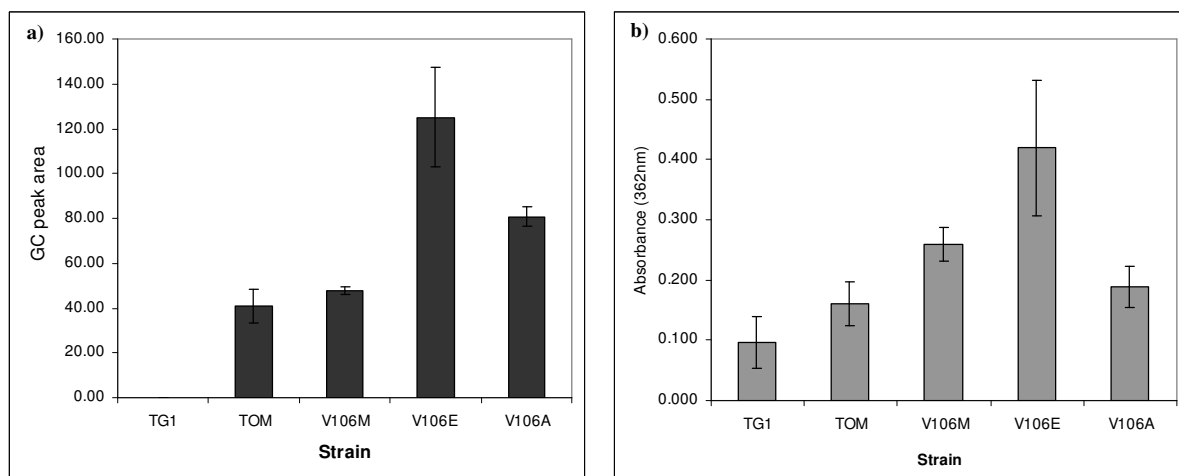


Figure 4

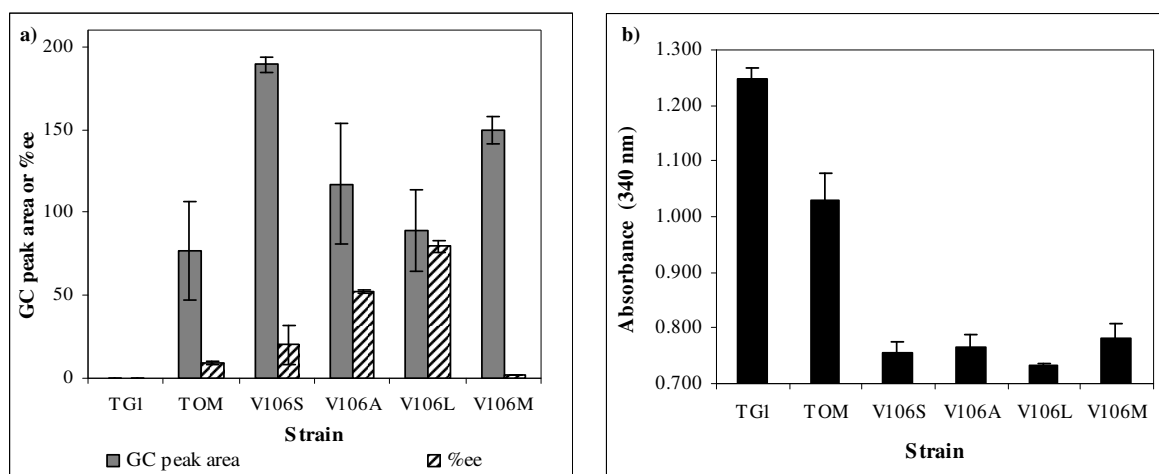


Figure 5