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Rapid Methods for High-Throughput Detection of Sulfoxides[▽]

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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 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, but no active variants have been 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 us to modify the method to a high-throughput format. The third method, selective inhibition of horse liver alcohol dehydrogenase, was used to rapidly screen highly active and/or enantioselective variants at position V106 of toluene *ortho*-monooxygenase in a saturation mutagenesis library, using methyl-*p*-tolyl sulfide as the substrate. A success rate of 89% (i.e., 11% false positives) 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 of 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 and inexpensive and do not require special equipment.

The growing demand for green catalytic processes has increased the utilization of enzymes as industrial biocatalysts for the synthesis of fine chemicals (6, 19, 20). 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 (23). 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 (32, 39).

Screening systems are divided into three different classes. The first class contains assays applicable to testing growing or resting microbial colonies for enzymatic activity directly on agar plates (23), for example, detection of epoxide hydrolase activity on butane oxide by use of safranin O. Oxidation of the 1,2-diol product by *Escherichia coli* modified the membrane potential and led to accumulation of the red dye in the colonies producing active enzyme (34). 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 (TMOs) for regioselective oxidation of substituted phenols (12, 30). The positive clones produced a red halo around the cells. These assays are high-throughput, simple procedures but often require a tailored substrate with a chromophore, such as bromonaphthol or azo-dye (23).

The second class includes chromogenic and fluorogenic assays applicable in microtiter plates or microarray formats (23). Microtiter plates in 96- or 384-well format are particularly well suited for spectroscopic reading using either UV-visible or fluorescence plate readers. This class may be subdivided into the following four groups: (i) enzyme-coupled assays, such as the determination of dehydrogenase activity through formation of NADH from NAD and an absorbance change at a wavelength of 340 nm; (ii) assays using chromogenic and fluorogenic substrates, such as various synthetically labeled substrates that are commercially available for the determination of hydrolytic activity produced by lipases, phosphatases, glycosidases, amidases, etc.; (iii) assays using 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) microarray assays using a solid support, enabling screening of thousands of samples. The high-throughput potential of these methods was demonstrated by profiling of 40 different esterases and lipases across 35 different fluorogenic ester substrates, using only 50 μl of each enzyme solution and a submilligram quantity of each substrate for over 7,000 tests (2).

The third class of enzymatic assays rely on product detection by instruments and include gas chromatography (GC), high-pressure liquid chromatography (HPLC), mass spectrometry, nuclear magnetic resonance (NMR) spectrometry, and infrared radiation assays that have been adapted for high throughput (22, 23, 33). Such assays require expensive and sophisti-

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cated equipment, but they allow working directly with the substrate of interest and are rapidly adapted once the instrument is available (23).

Various chemical substances can be synthesized by bacteria and fungi, among which are the chiral sulfoxides (5, 10, 11, 24, 36). As natural products, chiral sulfoxides possess a wide range of biological activities, from flavor and aroma precursor activities to antimicrobial properties. In addition, they are efficient auxiliaries that lead to essential asymmetric transformations (3, 11). Furthermore, one of the most significant applications of chiral sulfoxides is in the pharmaceutical industry (3). The world's best-selling antiulcer 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 TMOs. Three of the four methods were exploited successfully to a 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 (28), was useful for detecting mutants with high activity and enantioselectivity in the oxidation of methyl *p*-tolyl sulfide.

MATERIALS AND METHODS

Chemicals. Thioanisole (methyl phenyl sulfide; 99%), methyl *p*-tolyl sulfide (99%), methyl phenyl sulfoxide (97%), (*R*)- and (*S*)-methyl *p*-tolyl sulfoxide (98%), liver acetone powder (LAP; 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). Trifluoroacetic acid anhydride (TFAA) was purchased from Riedel-de Haën (Seezle, Germany). Sodium iodide was purchased from Spectrum (New Brunswick, NJ). Omeprazole-sulfide was received from the TAMI-IMI Institute for R&D (Haifa, Israel). (*S*)-Omeprazole was obtained from Teva (Petach-Tikva, Israel). All materials used were of the highest purity available and were used without further purification.

Bacterial strains and growth conditions. *Escherichia coli* TG1 (*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) carrying plasmid constructs was routinely cultivated at 37°C with shaking at 250 rpm in a TU-400 incubator shaker (orbital shaker incubator; MRC, Holon, Israel) in Luria-Bertani (LB) medium (26) supplemented with kanamycin at 100 μg/ml (LBK) to maintain the plasmids. To stably and constitutively express the TMO genes from the same promoter, the expression vectors pBS(Kan)TOM (7), pBS(Kan)TpMO (31), pBS(Kan)ToMO (35), and pBS(Kan)T4MO (31) were constructed as described earlier. Expression of TMOs (wild type [WT] and protein variants) by pBS(Kan) vectors within *E. coli* strains produced blue or brown colonies on agar plates and in broth cultures. The color is indicative of indigoid compounds formed by the oxidation of indole from tryptophan (9, 25).

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 VP 381 library copier (V&P Scientific, Inc., San Diego, CA), and transferring them to 96-deep-well plates with plastic lids (ABgene; Thermo Fisher Scientific, Epson, United Kingdom) containing 1.2 ml of LBK per well. The cells were grown for 18 h at 37°C with shaking at 250 rpm in a TU-400 incubator shaker, followed by centrifugation at 3,000 × *g* for 10 min at 25°C, using a Sigma 4K15 centrifuge (Sigma, Osterode, Germany). The cell pellets were resuspended in 350 μl of 20 mM phosphate buffer (PB), pH 7, and then 100 μl was removed to measure the cell density (optical density [OD] at 600 nm, ~1.1). The substrate omeprazole-sulfide was added to 250 μl 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 stopped by additional centrifugation at 3,000 × *g* for 10 min at 25°C. One hundred microliters of the supernatant was transferred to F96 MicroWell black microtiter plates (Nunc, Roskilde, Denmark). Thirty microliters of 0.1 N HCl was added to each well. The plate was incubated for 5 min at room temperature, and the fluorescence signal was measured (excitation wavelength, 370 nm; emission wavelength, 560 nm) using a Synergy HT multiplate reader (BioTek Instruments, VT). All liquid handling steps were conducted using an epMotion 5070 robotic system (Eppendorf AG, Hamburg, Germany).

(ii) Adrenaline test. Omeprazole-sulfide, thioanisole, and methyl-*p*-tolyl sulfide were examined as potential substrates in the adrenaline 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 × *g* for 10 min at 25°C (Sigma 4K15 centrifuge). The cell pellets were resuspended in 2.5 ml of 20 mM PB, pH 7, to a 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 × *g* for 1 min), using a Minispin centrifuge (Eppendorf, Hamburg, Germany). Eight hundred fifty microliters of the supernatant was transferred to a new vial and then incubated with 50 μl $NaIO_4$ (10 mM stock in water) for 20 min. Subsequently, 150 μl of adrenaline-hydrochloride (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 1,050 μl.

(iii) TFAA-NaI test. Bacterial growth was performed as described for the fluorescence test. The growth was followed by centrifugation at 3,000 × *g* for 10 min at 25°C, using a Sigma 4K15 centrifuge (Sigma, Osterode, Germany). The cell pellets were resuspended in 600 μl of 0.1 M PB, pH 7, and then 100 μl was removed to measure the cell density (OD at 600 nm, ~1). The substrate, methyl phenyl sulfide, was added to 500 μl 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, Nurenberg, Germany), at 25°C. The reaction was stopped by additional centrifugation at 3,000 × *g* for 10 min at 25°C. Four hundred microliters of the supernatant was transferred to a new polypropylene deep-well plate (ABgene; Thermo Fisher Scientific, Epson, United Kingdom), and 400 μl of ethyl acetate was added to each well (1:1 [vol/vol]) for the extraction of reactants. After vigorous pipetting of the reaction mixture, phase separation was facilitated by an additional centrifugation step (3,000 × *g* for 10 min), and 100 μl of the organic phase was transferred to a new polypropylene 96-well plate (Grenier) with a flat bottom (Sigma-Aldrich, Rehovot, Israel). TFAA (10 μl from a 0.5 M stock in acetone) and sodium iodide (NaI) (100 μl from a 0.5 M stock in acetone) were added, followed by vigorous pipetting and a 2-min incubation at room temperature. The total assay volume was 210 μl. Determination of the amount of iodine released during the reduction reaction was measured at 362 nm in a multiplate reader (Molecular Devices, Sunnyvale, CA).

(iv) Selective inhibition of HLADH. Bacterial growth conditions were as described for the fluorescence test, utilizing a VP 381 library copier and an epMotion 5070 liquid handling system. The cell pellets were resuspended in 350 μl of 46 mM sodium phosphate buffer, pH 7, to a final OD of 1.1. The substrate methyl *p*-tolyl sulfide was added to 250 μl of bacterial suspension (100 μl of the suspension was 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 4 h with shaking at 600 rpm, using a Vibramax 100 shaker, at 25°C. The reaction was stopped by additional centrifugation at 3,000 × *g* for 10 min at 25°C, using a Sigma 4K15 centrifuge. Ninety microliters of supernatant from each well was transferred to a new 96-well polystyrene plate (Nunc, Roskilde, Denmark). Ten microliters of freshly prepared LAP solution (from a 10-mg/ml filtered stock solution in 46 mM ice-cold sodium phosphate buffer, pH 7) was added. The reaction was initiated by adding 90 μl of freshly prepared 100 mM ethanol and 30 mM β-NAD in 46 mM ice-cold sodium phosphate buffer, pH 7. The total assay volume was 190 μl. Absorbance data at a wavelength of 340 nm were collected over a period of 180 min, using a multiplate reader (Molecular Devices, Sunnyvale, CA).

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

Analytical methods. Conversion of thioanisole and methyl *p*-tolyl sulfide to the corresponding sulfoxides was determined with a GC 6890N instrument (Agilent Technologies, Santa Clara, CA), using a 30-m by 0.32-mm by 0.25-μm capillary

TABLE 1. Primers used for random mutagenesis via epPCR, for sequencing of *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 (5′–3′)
Mutagenesis primers	
T4MObefEcoRI Front	CCATGATTACGCCAAGCGCG
T4MOABRear	TCCATGCTCTTCACTGTTGAC
BM_T4MO_285_Front	GGATTACTACACGCCGTTGGAGNNNCGCAGCCAG
T4MO_285_Rear	AACTCCTTGAATGACTGGCTGCGNNNCTCCAACGG
TOM_FRONT_ep_np	CTCCAGCAAATCCACATCAACC
TOM_REAR_ep_np	GGGGCTGAATGTTGAGTTGATAGG
Sequencing primers	
T4MObefEcoRI Front	CCATGATTACGCCAAGCGCG
T4MO Seq1	CCCGCATGAATACTGTAAGAAGGATCGC
T4MO Seq2	GCTCGTTGATAGATCTGGGCTTGGAC
T4MO Seq3	AATCTATTGAAGAGATGGGCAAAGACGC
TOM_FRONT_ep_np	CTCCAGCAAATCCACATCAACC
TOM_BSIWI_FRONT (TOM1)	CCGATGGAGAAAGTGTTTCCGTACGAC
TOM Seq 3	CGCCACATGACACTCGGCATC
TOM seq 4	CGGCGACAAGTATCACTTCTGC

column packed with γ-cyclodextrin trifluoroacetyl (Chiraldex G-TA; ASTEC, Bellefonte, PA) and a flame ionization detector. The temperature for thioanisole was programmed as follows: T_1 = 110°C (dT/dt = 10°C/min), T_2 = 130°C (dT/dt = 20°C/min), and T_3 = 160°C (13 min), with a split ratio of 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 sulfoxide. For determination of methyl *p*-tolyl sulfide conversion, the temperature was programmed as follows: T_1 = 110°C (dT/dt = 10°C/min), T_2 = 130°C (dT/dt = 20°C/min), and T_3 = 160°C (17.5 min), with a split ratio of 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 with an Agilent 1100-series instrument (Agilent Technologies, Santa Clara, CA) using an Eclipse XDB C_{18} column (5 μm, 4.6 by 150 mm; Agilent Technologies). The samples were diluted in H_2O-acetonitrile (75%–25%) and filtered using a 0.45-μm polyvinyl difluoride filter (Millex HV; Millipore, Cork, Ireland). The mobile phase was set as 70%–30% H_2O-acetonitrile for 0 to 1 min, with a gradient to 40%–60% H_2O-acetonitrile at 6 min and a gradient to 70%–30% H_2O-acetonitrile at 12 min, and the flow rate was 1 ml/min. Compounds were identified by comparison of retention times (omeprazole-sulfide retention time, 7.1 min; esomeprazole retention time, 4.8 min) and UV-visible spectra to those of authentic standards. Calibration curves were 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 PCR. Two primers, BM_T4MO_285_Front and T4MO_285_Rear (Table 1), were designed to 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 Biolabs, Ipswich, MA) was used in the PCR to minimize random point mutations, and pBS(Kan)T4MO was used as the template. The first, 1,006-nucleotide degenerate fragment was amplified by PCR using two primers, T4MObefEcoRI Front and T4MO_285_Rear, and the second degenerate fragment (1,062 nucleotides) was amplified by PCR using two other primers, T4MO_285_Front and T4MOABRear. The PCR program consisted of an initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 2.2 min, with a final extension at 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 (2,034 bp), using T4MObefEcoRI Front and T4MOABRear as primers. The PCR program was programmed as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 3.15 min, with a final extension at 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 plasmid library was transformed into *E. coli* TG1 competent cells via electroporation using a MicroPulser electroporator (Bio-Rad, Hercules, CA).

Random mutagenesis via epPCR. The *tmoA* and *tmoB* genes, encoding α and γ hydroxylase subunits, respectively, and 20% of the *tmoC* gene (1,936 bp) in pBS(Kan)T4MO were amplified using error-prone PCR (epPCR). The 100-μl reaction mixture contained 67 mM Tris-HCl (pH 8.8 at 25°C), 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 5 mM $MgCl_2$, 0.35 mM $MnCl_2$, 1 M betaine, 40 ng of template DNA [pBS(Kan)T4MO], a 0.2 mM concentration of dATP and dGTP, a 1 mM concentration of dCTP and dTTP, 5 U of *Taq* DNA polymerase (Bioline; Biotaq, London, United Kingdom), and 30 pmol of each primer (T4MObefEcoRI Front and T4MOABRear) (Table 1). The T4MObefEcoRI Front primer is targeted upstream of the EcoRI restriction site, located in the multiple cloning site, and the T4MOABRear primer is targeted downstream of the naturally occurring AatII site within the *tmoC* gene.

The *tomA3* and *tomA4* genes, encoding α and γ hydroxylase subunits, respectively, in pBS(Kan)TOM (2,128 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 targeted upstream of the BlpI restriction site, and the TOM_Rear_ep_np primer is targeted downstream of the MfeI restriction site. The PCR program used for DNA amplification consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 59.3°C or 58.3°C (for T4MO or TOM, respectively) for 1 min, and 72°C for 2.5 min, with a final extension at 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 BlpI and MfeI (New England Biolabs, Beverly, MA), respectively, replacing the corresponding fragment in the original plasmid. The resulting plasmid library was transformed into *E. coli* TG1 competent cells via electroporation, using a MicroPulser electroporator.

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, with emission at 560 nm (29) (Fig. 1a). Under the experimental conditions used, omeprazole-sulfide remains inactivated and does not interfere with the fluorescence signal (Fig. 2a). A calibration curve prepared with buffer containing 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 (Fig. 2b). In order to establish optimal conditions for the assay, several parameters were examined, including sample volume, assay

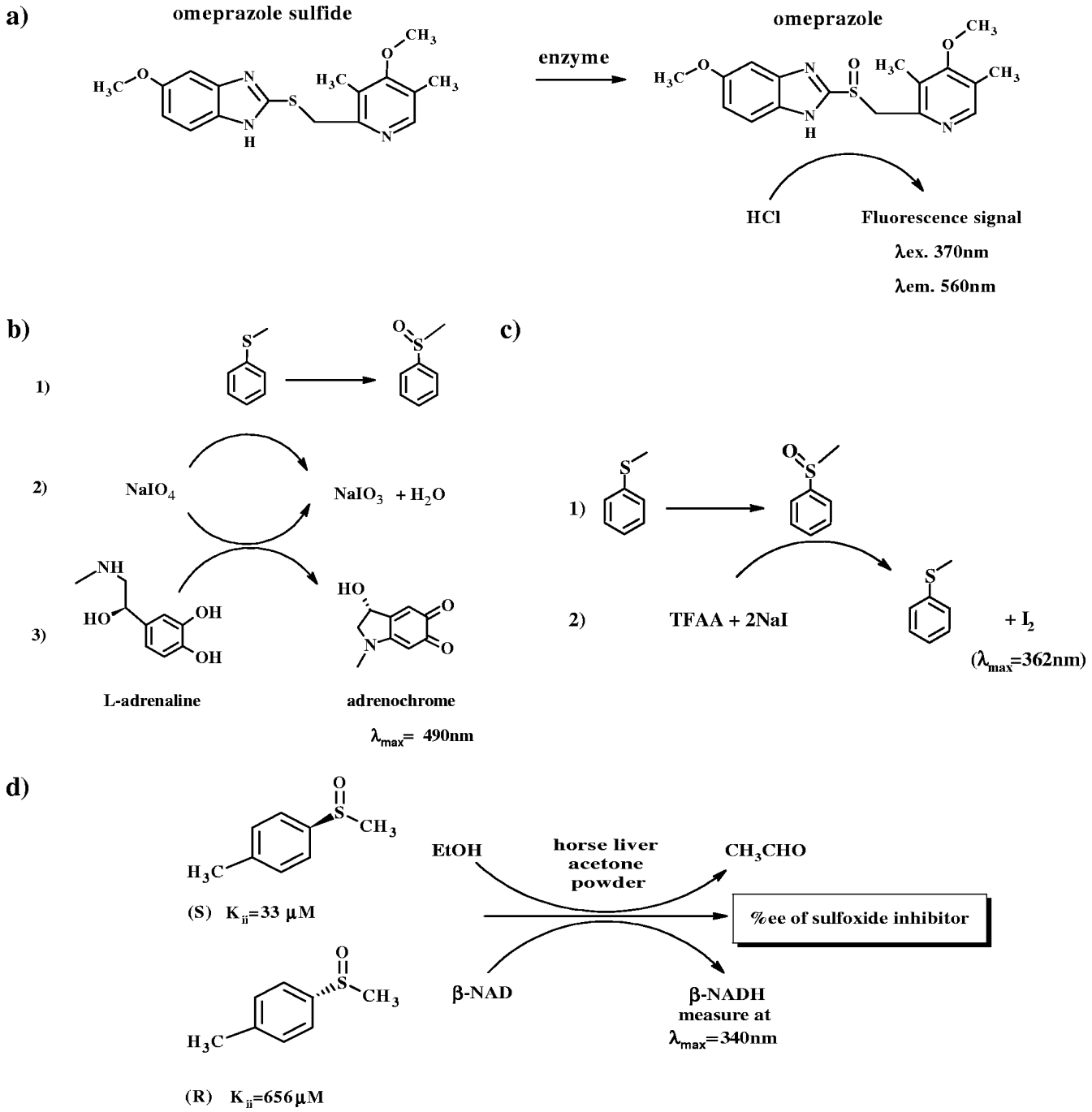

FIG. 1. Chemical reactions used to detect sulfoxides in this study. (a) Fluorescence-based assay for direct measurement of the omeprazole-HCl complex. (b) Adrenaline-based assay for measurement of the remaining sulfide in the reaction. (Adapted from reference 38.) (c) TFAA-NaI reagent for the reduction of sulfoxide and liberation of I_2. (d) Selective inhibition of horse LAP by methyl *p*-tolyl sulfoxide. (Adapted from reference 28.)

medium (LBK or PB), ionic strength of the biotransformation buffer, and the pH of the sample, adjusted with HCl (Fig. 2a). Eventually, this technique was modified for high-throughput screening of bacterial libraries in 96-well microtiter plates, using the 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 microtiter plates, and the addition of HCl, followed by incubation for 5 min and fluorescence measurement using a multiplate reader. The use of LBK medium for the biotransformation resulted in a high fluorescence signal and interfered with the results (Fig. 2a); therefore, the biotransformation was performed in buffer and the cells were removed prior to the addition of HCl. PB at an ionic strength of 100 mM was

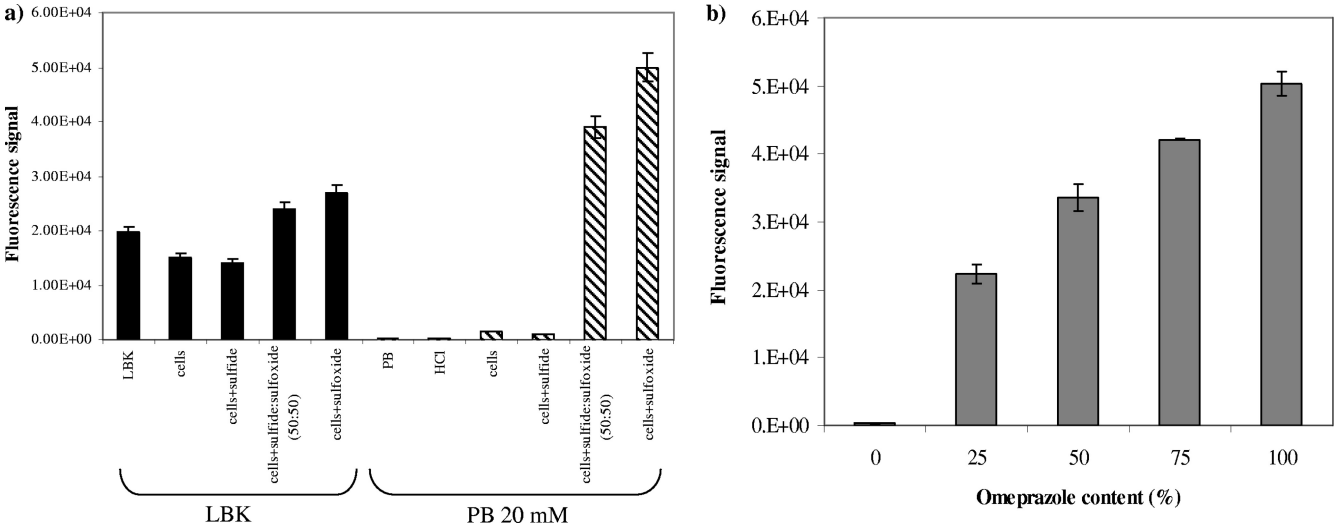

FIG. 2. Fluorescence-based assay for direct measurement of HCl-activated omeprazole. (a) Fluorescent signals of different treated samples of omeprazole and omeprazole-sulfide (total concentration of 0.5 mM). Samples including cells and standards in LBK or 20 mM PB were centrifuged, and the supernatant was examined for fluorescence after activation with 1 N HCl. (b) Calibration curve of omeprazole in 20 mM PB, obtained from the multiplate reader at an excitation wavelength of 370 nm and an emission wavelength of 560 nm. Every bar in the graph represents at least three independent experiments (the total concentration of substrate and product was 0.5 mM).

inappropriate for the assay (results not shown), but 20 mM was adequate, as depicted for the control sample (Fig. 2a). The optimal pH for the reaction was determined to be 2.

Random libraries of T4MO and TOM were prepared using epPCR, as were three saturation mutagenesis libraries for positions TOM V106 and 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 prime targets here. For instance, T4MO I100A and I100S mutants were highly active in the hydroxylation of nitrobenzene (12), whereas an I100G mutant oxidized methyl *para*-tolyl sulfide 11 times faster than the WT 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 above; however, no active variants on omeprazole-sulfide were found.

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. The adrenaline test is a back-titration procedure which measures the concentration of periodate-sensitive substances (38). In the original publications, diols and epoxides were used as substrates (37, 38). In the present TMO-catalyzed reaction, sulfoxide was produced using whole cells, and the remaining sulfide was oxidized with excess sodium periodate, followed by reaction of the remaining periodate with adrenaline to form the red dye adrenochrome (Fig. 1b). It is expected that the more sulfoxide is produced by the cells, the more intense the red color will be (less sulfide remains, and 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 $NaIO_4$ and adrenaline-hydrochloride, ionic strength of pH 7.0 PB, and concentration of $NaIO_4$. It was found that the reaction cannot be performed with 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 PB influences the absorbance (the absorbance is higher when 20 mM PB is used than when 100 mM PB is used). Finally, it was discovered that the presence of cells interferes with absorbance measurement; therefore, an additional centrifugation step is needed to obtain cell-free medium (results not shown).

The following 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 correlations between absorbance at 490 nm and increasing sulfoxide concentrations (Fig. 3). But when the method was tested on WT TMOs and their variants, the results did not correlate with those of 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 (Fig. 3); hence, this method was found to be inappropriate as a high-throughput screening exam.

(iii) TFAA-NaI test. The TFAA-NaI method is based on the use of a TFAA-NaI mixture as a reducing agent, con-

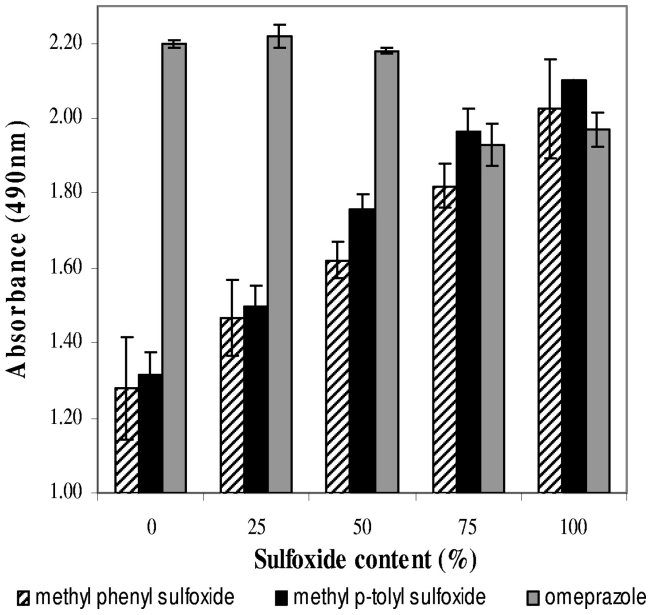

FIG. 3. Adrenaline-based assay for measurement of the remaining sulfide in the biotransformation reaction. Calibration curves created with 20 mM PB (without the presence of cells) are presented for methyl phenyl sulfoxide, methyl *p*-tolyl sulfoxide, and omeprazole at a wavelength of 490 nm. Every bar in the graph represents at least three independent experiments (the total concentration of substrate and product was 1 mM).

verting sulfoxides to the corresponding sulfides, followed by the release of iodine, whose yellow color is measured at 362 nm (17) (Fig. 1c). There is a direct correlation between sulfoxide production and the 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, but this solvent did not extract the substrate and the product well and was found to damage 96-well polystyrene plates. A mixture of glacial acetic acid and acetic anhydride (95:5 [vol/vol]) 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 pH 7 PB 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 biotransformation. In addition, different concentrations and ratios of TFAA and NaI were examined (15 to 100 mM and 0.15 to 1 M, respectively). Eventually, the procedure consisted of 400 μl ethyl acetate as an extractant, 20 mM TFAA, and 0.2 M NaI, with 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 of 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 in order to prevent the extraction of interfering indigoid colors present in the cells. Since the assay is performed with organic medium, it cannot be executed in 96-well polystyrene plates. Recently, solvent-resistant 96-well plates (made from polypropylene) were marketed by Sigma-Aldrich, enabling us to exploit this simple and sensitive method in a high-throughput format. The final version of the assay showed a high correlation with GC

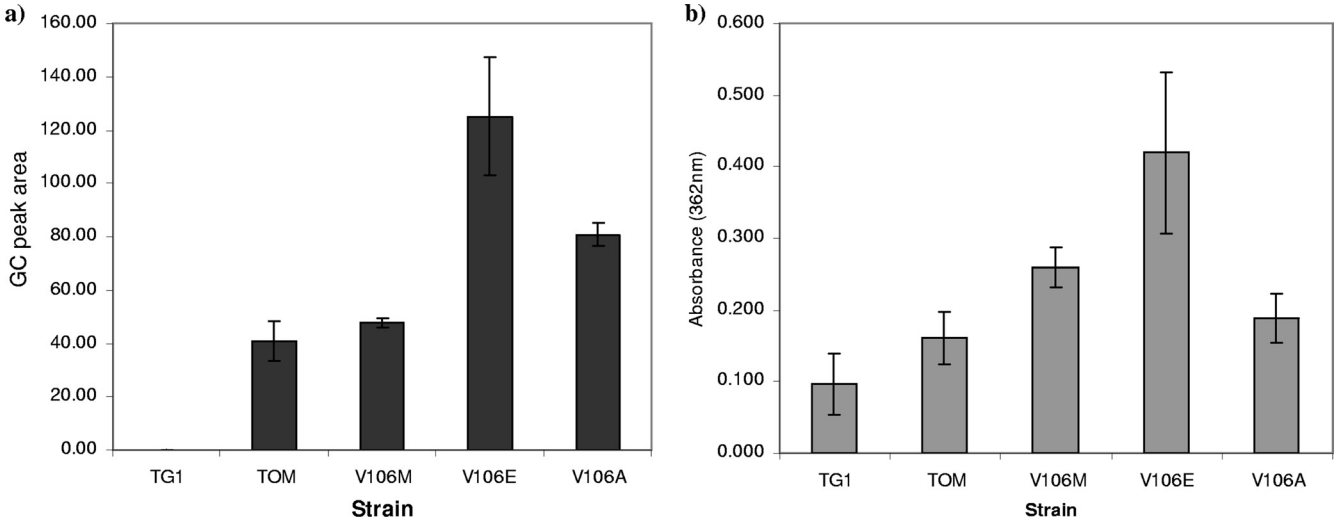

FIG. 4. Correlation of the TFAA-NaI test with GC measurements. TOM V106 variants as well as TG1 cells (without an insert, negative control) were examined by the two methods, using 1 mM methyl phenyl sulfide as a substrate. (a) GC measurements. (b) TFAA-NaI colorimetric measurements. Readings are normalized to cell density. Results represent averages for duplicates.

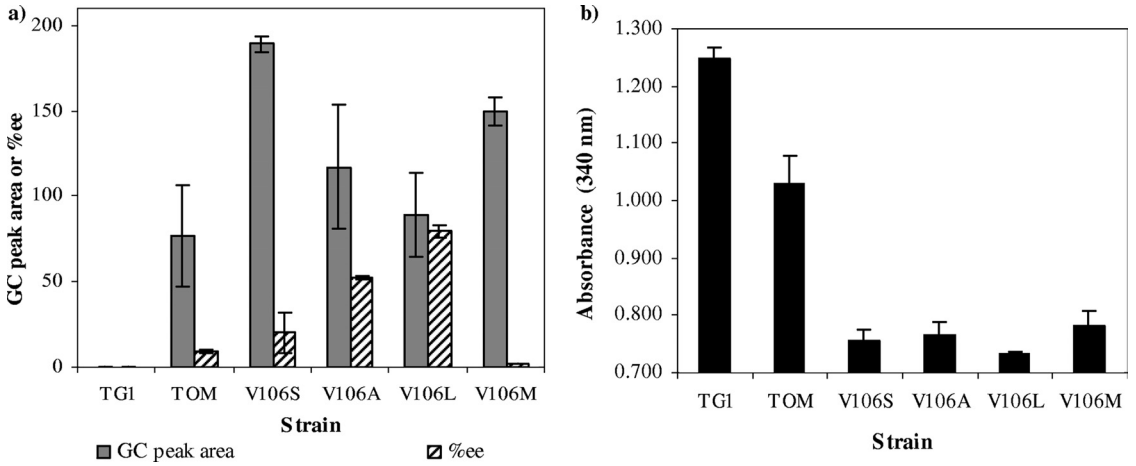

FIG. 5. Correlation of selective inhibition of HLADH with GC measurements. TG1 cells, TOM, and a few TOM V106 variants were examined by the two methods, using 1 mM methyl *p*-tolyl sulfide as a substrate. (a) GC measurements. (b) Selective inhibition of HLADH. Results represent averages for duplicates.

measurements performed in parallel (Fig. 4), indicating that the tested variants are more active than WT TOM.

(iv) Selective inhibition of HLADH. The assay using HLADH is a modification of the method described by Sprout and Seto (28) and is based on the different inhibition constants of (*S*)- and (*R*)-methyl-*p*-tolyl sulfoxide for 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 (Fig. 1d). Thus, as (*S*)-methyl-*p*-tolyl sulfoxide concentrations increase, absorption from β-NADH decreases. Following a few preliminary trials, it was discovered that HLADH is no longer available commercially. An alcohol dehydrogenase from baker's yeast was evaluated, but it was not inhibited by methyl *p*-tolyl sulfoxide, probably due to a different protein structure from that of the horse enzyme. Finally, crude horse LAP was used successfully in the assay. Various parameters were examined, such as the pH of the buffer solution (pH 7 or 8.8), the influence of the presence of sulfide in the reaction mix, the volume and concentration of LAP, background provided by other substances in the solution, different ratios of β-NAD and ethanol, various substrate concentrations, and appropriate durations of the biotransformation and 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 4 h. During this time, product is already formed and the evaporation of the 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 was previously screened on methyl phenyl sulfide in our lab (10). The five best mutants in that study were also evaluated on methyl *p*-tolyl sulfide so the results could be compared with the present screen. Initially, WT TOM, its variants, and TG1/pBS(Kan) (the host without the enzyme insert was used as a negative control) were analyzed using the HLADH inhibition-based assay. In parallel, each sample was injected into a GC (Fig. 5). The negative control showed the highest absorbance signal, since no sulfoxide was produced. Mutants with higher activity and/or selectivity toward the (*S*)-sulfoxide than that of WT TOM exhibited lower absorbance due to strong inhibition of the HLADH. A 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 mutants and one worst-performing mutant were picked from each 96-well plate and analyzed using GC, making a total of 35 variants (best performing = the lowest absorption at 340 nm; worst performing = the highest absorption at 340 nm). Among the seven worst-performing variants chosen, five were indeed inactive and two were false negatives. Samples referred to as false-negative samples are variants that were active according to GC analysis but showed unexpectedly high absorbance at 340 nm in the screening assay. Among the 28 best-performing variants chosen, 24 were more enantioselective than the WT and 25 were more active. Three variants were false-positive samples, meaning that they were inactive according to GC analysis 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, the V106S mutant, and one of the most enantioselective variants, the V106A mutant, found in our previous 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 HLADH inhibition

enables us to select better variants which are either more enantioselective, more active, or both.

DISCUSSION

Chiral sulfoxides play a significant role in pharmaceuticals and in organic synthesis, as functionalized substances and as important chiral auxiliaries (3, 11, 15). In addition, there is a growing demand for green catalytic processes (19, 27), and therefore there is a continuous search for novel and better-performing biocatalysts. The 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 produce a wide spectrum of significant substances (21, 23). 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 (23). A suitable method for measuring sulfoxide formation or enantioselectivity has not yet been published according to our knowledge.

An appropriate screen should be able to detect and quantify sulfoxides in the presence of sulfides and to 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, including a fluorescence test for omeprazole, an adrenaline test, a TFAA-NaI test, and an HLADH inhibition test 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 assay, were exploited for high-throughput screening of TMO libraries and showed good correlations with HPLC or GC results.

It was previously reported by Morii et al. that acid activation of omeprazole provides a strong fluorescent signal (18). 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 PB. 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 workup steps, but the fluorescence signal was again high and caused a disturbing level of 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 (39). 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 (39). 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*)-omeprazole, this assay may be of use for screening other potential oxidizing enzymes.

The adrenaline test for enzyme catalysis, which was developed by Reymond and coworkers (38), is a versatile high-throughput assay used for detection of 1,2-diols, 1,2-amino alcohols, 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). Consequently, 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 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 biotransformation and oxidized by sodium periodate. The Z′ factor calculated for the adrenaline test is 0.18, which indicates a low sensitivity. Therefore, it can be used only for determination of the presence or absence of sulfoxides, 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 and commercially available reagents and provides a 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 0.52, which indicates a good sensitivity. The availability of 96-well polypropylene plates enabled this method to be adapted successfully for a high-throughput format. This method is currently being evaluated in our lab with other prochiral sulfides.

The use of enzyme inhibition to measure the enantiomeric excess of a chiral sulfoxide was initially proposed by Sprout and Seto (28) 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 h. Similar to other fluorogenic and chromogenic methods for determination of enantioselective substances, such as assays for *p*-nitrophenol chiral esters, the HLADH inhibition test may be considered a high-throughput assay (21). Moreover, the calculated 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 for a wide range of sulfoxides, including chiral sulfoxides (8). For example, 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-β1 class 1 alcohol dehydrogenase from humans are 1,100 and 1,800 μM, respectively (8). In this work, it was also shown that the purity of the test enzyme is not crucial, as both pure HLADH and crude LAP 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 to 3 weeks, similar to the colorimetric method described by Bottcher and Bornscheuer for whole cells expressing esterases (4).

The only drawback of the HLADH inhibition method is the difficulty in distinguishing between high enantioselectivity (e.g., the V106M mutant) (Fig. 5) and a high conversion rate (e.g., the V106L mutant) (Fig. 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.

Although chromatography and NMR techniques for high-throughput screening have been developed in recent years (22, 23), they require expensive equipment, and data analysis is time-consuming. The methods shown in the present work are simple and inexpensive and do not require special equipment besides a multiplate reader.

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