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Regular Article

Whole-cell dependent biosynthesis of N- and S-oxides using human flavin containing monooxygenases expressing budding yeast

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ABSTRACT

Flavin containing monooxygenases (FMOs) represent one of the predominant types of phase I drug metabolizing enzymes (DMEs), and thus play an important role in the metabolism of xeno- and endobiotics for the generation of their corresponding oxides. These oxides often display biological activities, however they are difficult to study since their chemical or biological synthesis is generally challenging even though only small amounts are required to evaluate their efficacy and safety. Previously, we constructed a DME expression system for cytochrome P450, UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT) using yeast cells, and successfully produced xenobiotic metabolites in a whole-cell dependent manner. In this study, we developed a heterologous expression system for human FMOs, including FMO1–FMO5, in *Saccharomyces cerevisiae* and examined its N- and S-oxide productivity. The recombinant yeast cells expressed each of the FMO successfully, and the FMO4 transformant produced N- and S-oxide metabolites at several milligrams per liter within 24 h. This whole-cell dependent biosynthesis enabled the production of N- and S-oxides without the use of the expensive cofactor NADPH. Such novel yeast expression system could be a powerful tool for the production of oxide metabolites.

1. Introduction

Xenobiotics such as small molecular drugs, environmental pollutants, and dietary polyphenols are preferably metabolized by drug metabolizing enzymes (DMEs). This results in a decreased biological activity as well as increased water solubility of these xenobiotic compounds, which allow for their faster elimination from the body. Flavin-containing monooxygenases (FMOs; EC 1.14.13.8) are one of the most important families of non-cytochrome P450 (non-P450) enzymes involved in phase I metabolism. Among 860 drugs surveyed, FMOs were found to contribute to the metabolism of about 5% and catalyze about 2% of the more than 4000 oxidoreduction reactions undergone by these drug [1].

FMOs catalyze the oxidative metabolism of a broad range of structurally diverse chemicals containing soft nucleophiles such as sulfur or nitrogen. For this purpose, they require flavin adenine dinucleotide (FAD) as prosthetic group, NADPH as cofactor, and molecular oxygen as cosubstrate. FMOs are present in all kingdoms of life including bacteria, fungi, and plants [2,3], and are located on the membranes of the endoplasmic reticulum in eukaryotes. Humans possess five functional FMO genes, designated as FMO1–FMO5 [4]. FMO1 is expressed in fetal liver [5] and adult kidney [6], while FMO2 is expressed in lung tissue [7]. In the case of human FMO2, two alleles have been described: FMO2*1 is a functional full length version that is found with high frequency in Sub-Saharan African populations [8], whereas the FMO2*2 gene codes for a sequence with a premature stop codon that results in a non-functional protein [7]. FMO3 is considered as the prominent functional FMO form expressed in adult human liver [9,10]. Mutations of FMO3 produce phenotypes associated with the inherited disorder

Abbreviations: FMO, flavin containing monooxygenase; DME, drug metabolizing enzymes; P450 or CYP, cytochrome P450; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; FAD, flavin adenine dinucleotide; UGDH, UDP-glucose dehydrogenase; PAPS, phosphoadenosine phosphosulfate; HPLC, high performance liquid chromatography; LC-MS/MS, HPLC-tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitor; MTS, methyl *p*-tolyl sulfide; BZD, benzydamine; MTSO and BZDO, oxidized forms of MTS and BZD.

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trimethylaminuria [11]. The FMO4 gene is expressed in several tissues, however its expression level is very low. Finally, FMO5 is expressed in adult human liver [9,10].

Unlike mammals, yeast (*Saccharomyces cerevisiae*) does not possess several FMO isoforms, but a single one called yFMO that does not accept xenobiotic compounds. On the other hand, yFMO aids the folding of proteins that contain disulfide bonds by catalyzing the O_2 and NADPH-dependent oxidation of biological thiols [12,13].

Throughout the drug discovery and development process, drug metabolites may serve as analytical references for structure elucidation. Furthermore, with the introduction of the metabolites in safety testing (MIST) guidelines by the US Food and Drug Administration in 2008, all metabolites present in the human metabolism at >10% relative to the parent compound have to be subjected to toxicity studies [14]. The chemical preparation of authentic drug metabolites often requires multiple synthetic steps including several protection and deprotection reactions of various functional groups. To circumvent this issue, we aimed at the generation of scalable mimics of single steps of phase 1 metabolism reactions in vitro. This could be accomplished with heterologously expressed human enzymes in the form of ready-to-use biocatalysts such as *E. coli* and yeast. The use of whole-cell catalysts is beneficial in many aspects, since the enzyme performing the actual biotransformation does not need to be isolated and purified, which saves time and costs. FMOs have been successfully expressed in *E. coli*, and the whole-cell bioconversion of drug metabolites was obtained by human FMO3 and generated human FMO2 [15,16].

Other heterologous expression systems have also been developed for DMEs such as cytochrome P450 (P450), UDP-glucuronosyltransferases (UGTs), and sulfotransferases (SULTs). *S. cerevisiae* offers several advantages for the expression of DMEs. More than three decades ago, Sakaki et al. [17] successfully expressed mammalian P450 in *S. cerevisiae* AH22 cells, and these recombinant yeast cells were reported to effectively metabolize drugs, environmental pollutants, flavonoids, and dietary components [17–21]. Following the description of the use of the *S. cerevisiae* AH22 strain to express UGT [22], we established a whole-cell system to obtain glucuronides without the need of adding UDP-glucuronic acid by simultaneously expressing UGT and UDP-glucose dehydrogenase (UGDH) [23]. Furthermore, we successfully synthesized sulfo-conjugates without adding the expensive cofactor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) using a SULT expression system in yeast [24]. The *S. cerevisiae* system was found to have a higher productivity than a similar system using the fission yeast *Schizosaccharomyces pomb* [25].

In the present study, we describe the expression of five human FMO isoforms (FMO1-5) in *S. cerevisiae* AH22 yeast cells as well as the potential use of this system in the production of N- and S-oxidants.

2. Materials and methods

2.1. Materials

Yeast nitrogen base with ammonium sulfate was purchased from MP Biomedicals (Santa Ana, USA). L-Histidine and midazolam were acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Zymolyase from *Arthrobacter luteus* was purchased from Seikagaku Corporation (Tokyo, Japan). Methyl *p*-tolyl sulfide and benzydamine hydrochloride were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Benzydamine *N*-oxide hydrogen maleate was obtained from Sigma-Aldrich Corp. (St. Louis, MO). Methyl *p*-tolyl sulfoxide was purchased from Apollo Scientific Ltd. (Manchester, UK).

Rabbit polyclonal anti-FMO1 (ab97720), anti-FMO2 (ab95977), and anti-FMO3 (ab126711) antibodies were purchased from Abcam (Cambridge, UK), while anti-FMO5 (GTX114414) was purchased from GeneTex, Inc. (Irvine, CA). Goat polyclonal anti-FMO4 (T14, sc-104258) was acquired from Santa Cruz Biotechnology Inc. (Dallas, USA). Alkaline phosphatase (AP)-conjugated goat polyclonal antibody to rabbit IgG (7054) was obtained from Cell Signaling Technology (Massachusetts, USA). AP-conjugated rabbit polyclonal antibody against goat IgG (A4187) was purchased from Sigma-Aldrich Corp. All chemicals and solvents were of the highest commercially available grade.

2.2. Construction of an FMO expression system in yeast

In order to construct an FMO expression system in yeast, the *Escherichia coli* (*E. coli*)—yeast shuttle vector pGYR was used. Such vector contains a glyceraldehyde-3-phosphate dehydrogenase promoter and terminator derived from *Zygosaccharomyces rouxii*, and has been used for P450 expression in previous studies [26]. Synthetic cDNA fragments were obtained from Thermo Fisher Scientific Inc. (Waltham, MA); they contained 15bp additional sequences at the 5′- and 3′-end, which are the homologous sequences to the 3′- and 5′-end of linearized pGYR by Hind III, respectively, and were optimized for codon usage in *Saccharomyces cerevisiae*. These cDNA fragments were ligated with linearized pGYR by Hind III using the In-Fusion HD cloning kit (TaKaRa Bio Inc., Otsu, Japan). GenBank information of human FMO genes were described below (FMO1; NM_001282692, FMO2; NM_001460, FMO3; NM_006894, FMO4; NM_002022, FMO5; NM_001461).

The transformation of *S. cerevisiae* AH22 yeast cells (ATCC 38626) was performed by using the lithium acetate method as previously described [23]. The resultant clones were genotyped by direct colony PCR using KOD FX Neo (Toyobo Co., Ltd., Osaka, Japan).

2.3. Cultivation of the recombinant yeast cells expressing FMOs

The cultivation of the recombinant yeast cells was performed as previously described [24]. A glycerol stock of FMO transformant was spread on SD + His agarose plates, which were preincubated at 30 °C for 2–3 days. After picking the colonies, the cells were cultivated in 1 L SD + His medium at 30 °C overnight upon shaking at 200 rpm. The cells were harvested at a cell density of 2.0–2.5 OD660 and subsequently resuspended in an equal volume of distilled water. The cells were stocked at −80 °C and used as biocatalyst after thawing.

2.4. Immunoblot analysis

Whole yeast proteins were analyzed by immunoblot analysis as previously described [23,24]. Yeast pellets were treated with 0.5 mg/mL zymolyase 20T (Nacalai Tesque, Kyoto, Japan) for 5 min at 30 °C, and then subjected to SDS-PAGE using 10% acrylamide gel. The resulting polypeptide bands were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Berkeley, CA, US), equilibrated with methanol, and subsequently blocked with 10% bovine serum albumin (BSA) in a TBS buffer at room temperature for 1 h. After incubation with primary and AP-conjugated secondary antibodies, respectively, the detection was performed using the BCIP-NBI solution kit for alkaline phosphatase stain, nuclease tested (Nacalai Tesque Inc., Kyoto, Japan).

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2.5. Whole-cell-dependent biosynthesis of N-oxides and sulfoxides

The enzymatic activities of the FMO transformants were evaluated using the universal substrates methyl *p*-tolyl sulfide (MTS) and benzydamine (BZD). The optimization of the reaction conditions was carried out varying the different reaction parameters, and the optimized conditions were as follows. Wet yeast cells were suspended at a concentration of 25% (w/v) in 100 mM KPi buffer (pH 8.3) containing 8% (w/v) glucose, and 10 mM substrate in DMSO was added to the cell suspension at a final concentration of 0.1 mM. The reactions were conducted at 30 °C with shaking. Following incubation, a 2.5-fold volume of chloroform:methanol (3:1, v/v) was added to the reaction medium containing the yeast cells. The extracts were clarified into an upper aqueous and lower organic phase by centrifugation (12,000×*g*, 10 min, room temperature). The lower organic phase was dried and resolved in 100 μl of the solvent used for the subsequent HPLC and LC-MS/MS analysis.

2.6. HPLC analysis

The rate of S-oxygenation of methyl *p*-tolyl sulfide in the yeast cells was determined by using a HPLC system consisting of a Waters 2695 separations module, Waters 2487 dual-λ absorbance UV detector, and Cosmosil 5C18-Ar-II column (5 μm, 4.6 × 150 mm; Waters, Milford, MA).

The gradient elution conditions were water–acetonitrile with 0.1% (v/v) formic acid, 10% acetonitrile (2 min), 10–80% acetonitrile (8 min), 80% acetonitrile (2 min), and 10% acetonitrile (3 min). The flow rate was set at 1.0 mL/min, the column temperature was 40 °C, and the injection volume was 10 μl. The formation of methyl *p*-tolyl sulfoxide was monitored at a wavelength of 254 nm, and the quantitation was carried out with authenticated standards.

2.7. LC-MS/MS analysis

The rate of N-oxygenation of benzydamine was analyzed by using a HPLC-tandem mass spectrometry (LC-MS/MS) system consisting of an Agilent 1200 series LC system (Agilent technologies Inc., Santa Clara, CA) coupled with an API 3200 QTRAP mass spectrometer (AB Sciex, Toronto, Canada) equipped with a Capcell Pak C18 UG120 column (5 μm, 4.6 × 250 mm; Shiseido, Tokyo, Japan). The isocratic mobile phase conditions were 20% acetonitrile containing 0.1% formic acid, the flow rate was set at 0.8 mL/min, the column temperature was 40 °C, and the injection volume was 2 μl.

The MS analysis was carried out by electrospray ionization (ESI) with a positive ion mode scan in a multiple reaction monitor (MRM) mode. MRM transitions were monitored independently corresponding to the precursor and product ion pairs for benzydamine N-oxide (*m/z* transition 326 → 102), benzydamine (*m/z* transition 310 → 86), and the internal standard midazolam (*m/z* transition 326 → 291). For the quantitative determination of benzydamine N-oxide, a calibration curve was prepared using the peak area ratio of the authentic standard to the internal standard over a concentration range of 0.1–30 μM.

3. Results

3.1. Establishment of a whole-cell dependent production system of N- and S-oxide metabolites using recombinant yeast cells expressing human FMOs

The expression level of the FMOs transfected in yeast was determined by immunoblot analysis using FMO-specific antibodies.

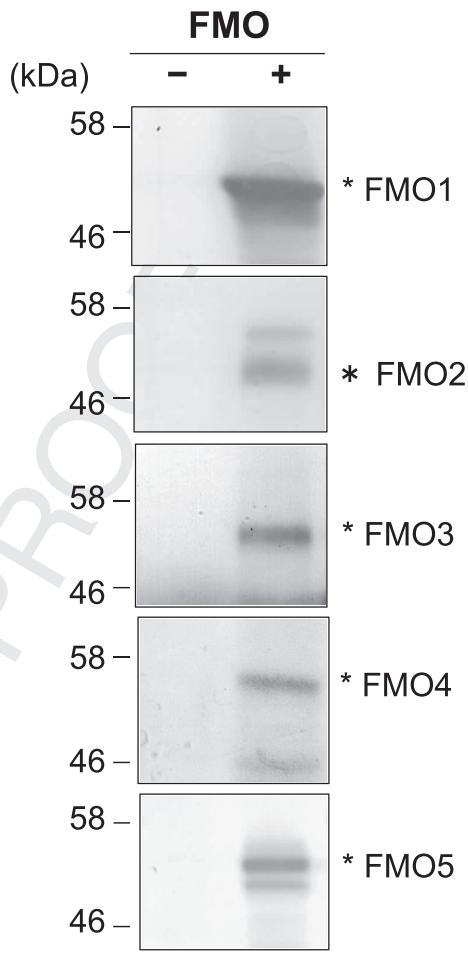

Fig. 1. Protein expression of human FMO transformants. Immunodetection was performed using anti-FMO1, FMO2, FMO3, FMO4, and FMO5 antibodies, respectively. The same amount of whole yeast protein (10 μg) from either the control pGYR vector alone (−) or human FMO transformed yeast strains (+) was loaded onto each lane. Q6

As shown in Fig. 1, the successful expression of the various FMO isoforms in *S. cerevisiae* AH22 cells was confirmed.

In order to determine the N- and S-oxygenation activities of the FMO transformants, methyl *p*-tolyl sulfide (MTS) and benzydamine (BZD) were used as universal substrates (Fig. 2a and Fig. 3a). MTS was successfully converted to its S-oxide form MTSO by FMOs (Fig. 2b). The FMO4 transformant efficiently catalyzed also the N-oxygenation of benzydamine (Fig. 3b). MTSO and BZDO were successfully generated in a whole-cell dependent manner (not only the FMO transformants, but also pGYR alone) without the need of adding an exogenous supplement of NADPH, suggesting that the endogenous NADPH present in the yeast cells was used by the FMOs to catalyze the N- and S- oxygenation reactions. Time-dependent conversions to MTSO and BZDO were performed using FMO4 and control pGYR transformants (Fig. 4). The time course analysis of MTS oxidation revealed that half of 0.1 mM MTS was converted to MTSO by FMO4 within 2 h (Fig. 4a).

3.2. Optimization of the reaction conditions for whole-cell dependent N- and S-oxide production

In order to improve the productivity of N- and S-oxide production in the yeast cells, the reaction conditions were optimized. The effect of the cell amount on the S-oxygenation was examined

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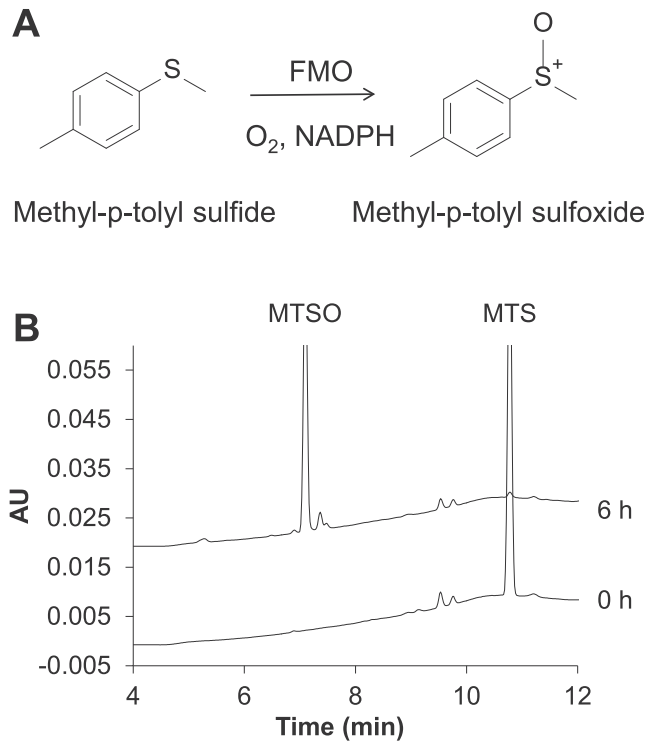

Fig. 2. S-oxygenation potential of the human FMO4 transformant towards methyl *p*-tolyl sulfide. (a) Reaction scheme of MTS S-oxygenation. (b) HPLC profile of 0.1 mM MTS incubated with the FMO4 transformant for 0 and 6 h.

using FMO3 and FMO4 transformants. As expected, the conversion rate of MTS to MTSO increased depending on the cell amount up to 25% (w/v) (Fig. 5a). Further increases of the cell amount led to a high viscosity of the reaction solution and low yield of MTSO. In addition, we examined the effect of the buffer pH on the N- and S-oxygenation, since FMO reactions are commonly conducted at a pH > 8.0 to ensure maximal FMO activity [27]. The benzydamine N-oxygenation catalyzed by FMO3 significantly increased when the reaction was conducted in a buffer at pH 8.3 compared with buffers at pH 6.8 and 7.4 (Fig. 5b). In contrast, the MTS S-oxygenation catalyzed by FMO4 was not significantly affected by changes in buffer pH (Fig. 5c).

As shown in Fig. 5, the FMO-expressing yeast showed an optimal conversion ratio towards MTSO and BZDO under the conditions of 8.3 buffer pH and 25% (w/v) yeast cells. We next compared the metabolic activity towards MTS and BZD among the five FMO transformants (Fig. 6). The oxidation activity towards MTS was confirmed for the FMO1, FMO3, and FMO4 transformants, with the highest activity observed for FMO4. The productivity per volume (L) is important from a practical standpoint, since the resultant N- and S-oxides are the purified by preparative HPLC. The highest productivity of MTSO and BZDO in the presence of the FMO4 transformant was 6.9 and 1.6 mg/L, respectively.

4. Discussion

The N- and S-oxides of some drugs are often formed by P450s and FMOs. The relative contribution of CYP vs FMO-dependent metabolism generally varies with the xenobiotic. Taniguchi et al. reported that N-oxygenation of BZD could be mainly catalyzed by FMO1/3 while N-demethylation could be catalyzed by P450 2D6 [27]. They suggested that the determination of the contribution of FMO isoforms to metabolism of new drug candidates might be paid careful attention.

Earlier, we developed DME expression systems expressing P450, UGT, and SULT using yeast cells, and successfully produced various metabolites of xeno- and endobiotics [17,23,24]. In the present study, we further constructed FMO expression systems in yeast. Subsequently, transformants expressing the FMO4 isoform were used to effectively produce several milligrams per liter of N- and S-oxide metabolites without the addition of NADPH. In vitro systems using subcellular fractions or purified FMO enzymes require an expensive cofactor (NADPH) for oxidation, therefore bypassing the requirement for exogenous NADPH offers a great advantage for the

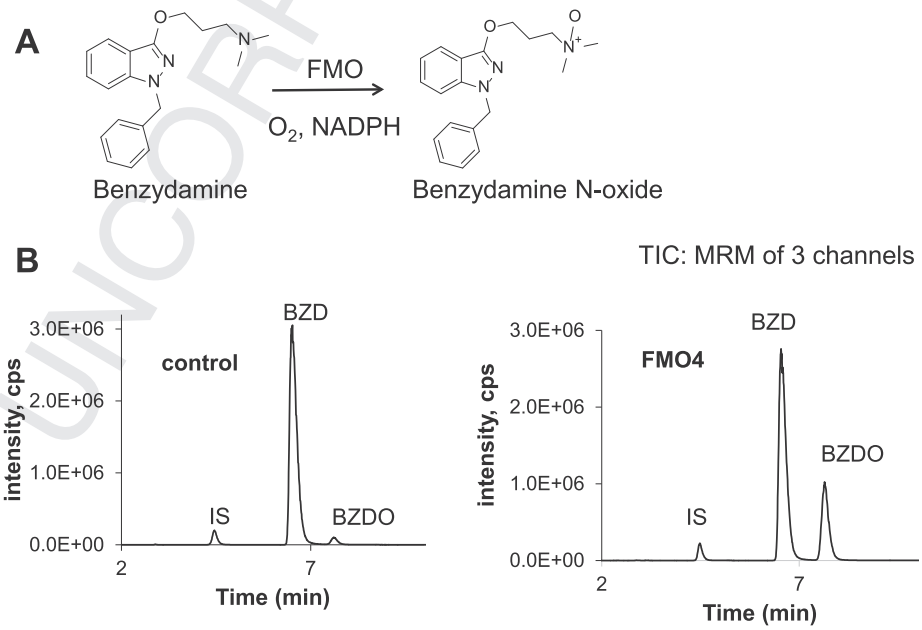

Fig. 3. N-oxygenation potential of the human FMO4 transformant towards benzydamine. (a) Reaction scheme of benzydamine N-oxygenation. (b) LC-tandem mass spectrometry quantitation using the MRM mode of 0.1 mM BZD incubated with the FMO4 transformant for 0 and 24 h. TIC chromatograms for the transitions *m/z* 310 → 86 (BZD), 326 → 102 (BZDO), and 326 → 291 (MDZ, IS). No analytical interference between the amine and N-oxide was detected. TIC, total ion current; MRM, multiple reaction monitoring.

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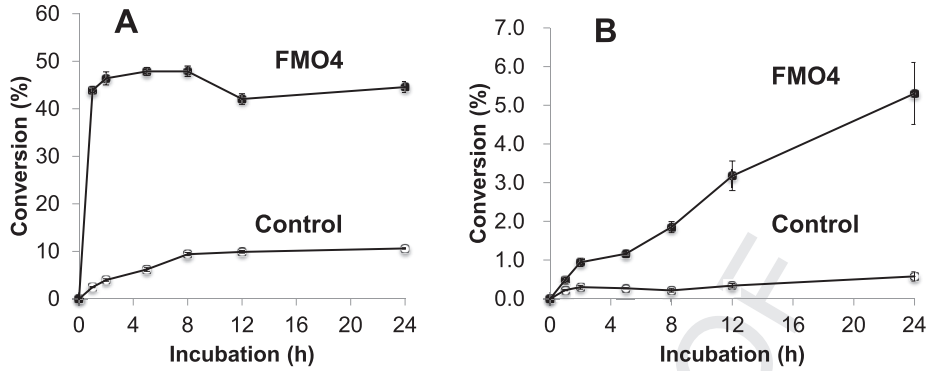

Fig. 4. Time-dependent conversion of MTS and BZD to MTSO (a) and BZDO (b) by the pGYR alone (open circle) and FMO4 transformant (filled circle). 0.1 mM of MTS and BZD were incubated with 25% (w/v) biomass. The values are shown as mean ± SEM (n = 3).

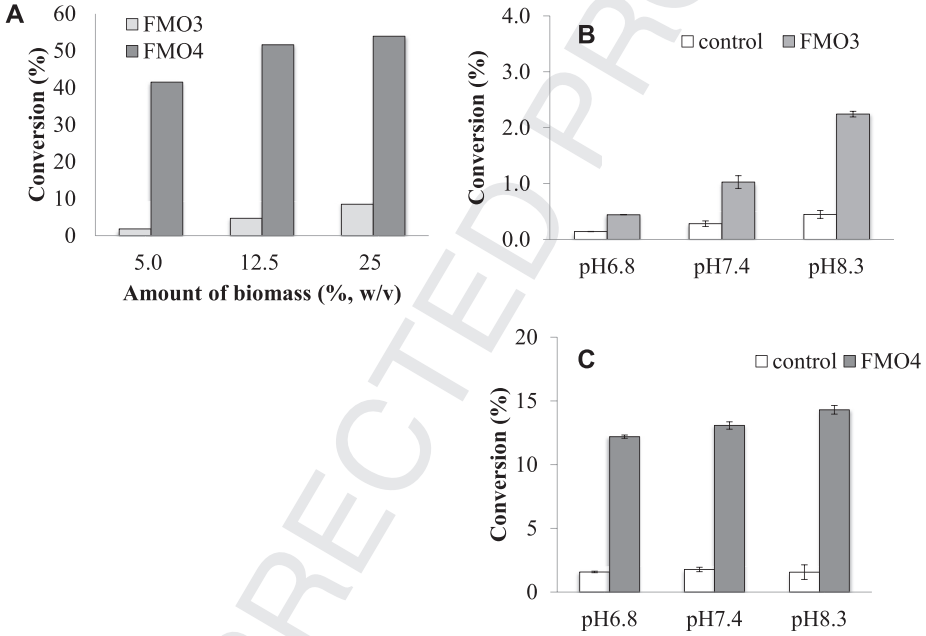

Fig. 5. Optimization of the reaction conditions using the MTS and BZD substrates. (a) Conversion of MTS to MTSO using different amounts of biomass. For each amount of biomass, 0.2 and 0.1 mM of MTS were incubated with the FMO3 and FMO4 transformant, respectively. The values are shown as mean ± SEM (n = 2–3). Effect of the buffer pH on the N-oxygenation of BZD (b) and S-oxygenation of MTS (c). 0.1 mM MTS and BZD was incubated with 10% (w/v) biomass of pGYR alone (open bar) and FMO3 or FMO4 (filled bar), respectively. The values are shown as mean ± SEM (n = 3).

industrial production of oxidants. Recently, Hanlon et al. [15] and Geier et al. [16] successfully produced N- and S-oxide metabolite using recombinant FMOs expressed in bacterial systems. Both *E. coli* and *S. cerevisiae* are considered suitable hosts for the whole-cell dependent production of N- and S-oxide metabolites owing to their ability to produce NADPH endogenously. In *S. cerevisiae*, FMO isoforms were expressed at a sufficient level without a concomitant reduction of inclusion body formation compared to bacterial expression systems, and whole-cell biosynthesis using frozen stocks of the recombinant yeast resulted in a stable expression along with good reproducibility.

The regio- or enantioselective production of metabolites is one of the greatest features of biosynthesis. In our previous study, we successfully produced conjugates of polyphenols in a regioselective manner. The regioselective oxidation ability of each FMO isoform towards ranitidine and phosphor-NSAID was reported [28,29]; therefore, it can be expected that our yeast expression system for FMOs could be used for predicting metabolic pathways of candidate compounds in humans.

In addition to this, drug metabolites are required as analytical standards for structure elucidation throughout the drug discovery and development process. Furthermore, 2008 MIST guidelines by the FDA established that all metabolites present at >10% relative to the parent compound in the human metabolism have to be subjected to toxicity studies [14]. Our yeast expression system for FMOs could be used in the production of oxide metabolites for quantitative analysis, however, improvements are necessary to attain sufficient amounts (~g order) of metabolites for toxicity studies.

In conclusion, the heterologous expression system for FMOs in yeast cells developed in this study offers two main advantages: (1) bypassing of the requirement for exogenous NADPH, (2) high recovery of N-and S-oxide metabolites from the yeast culture medium through a relatively simple procedure. Therefore, this FMO expression system could be useful for revealing the biological activity of FMO-dependent metabolites, and by combining it with the P450, UGT, and SULT expression systems, it can be envisaged to accelerate the study of the metabolism and involved metabolites.

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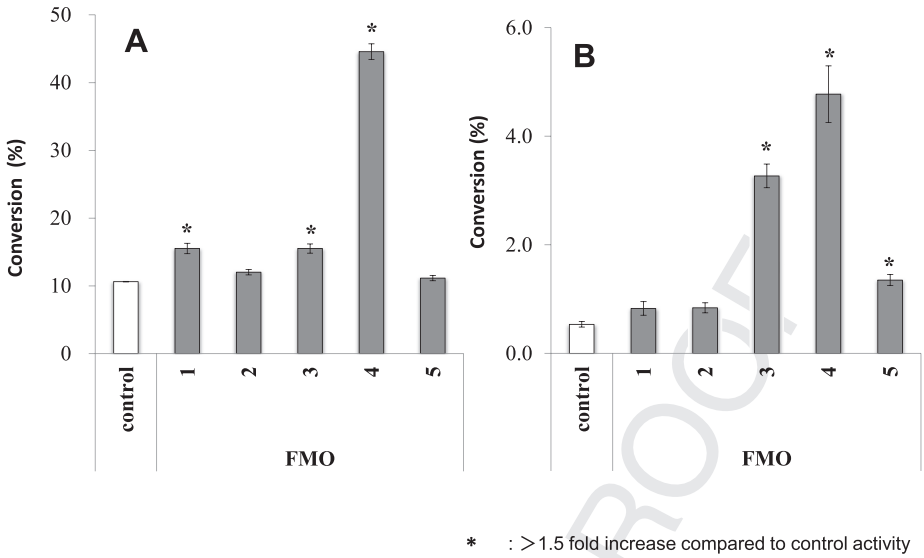

Fig. 6. Conversion of MTS and BZD to MTSO and BZDO, respectively, in each FMO transformant. 0.1 mM MTS (a) and BNZ (b) were incubated with the pGYR alone (open bar) and FMO transformant (filled bar). The values are shown as mean ± SEM (n = 3).

Authors contributions

Y.M. T.S., and S.I. designed the research and wrote the paper; Y.M., M.N., K.Y., and S.I conducted the experiments and analyzed the data. S.I. had primary responsible for the final contents.

Declaration of competing interest

There are no conflicts of interest to declare.

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