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Improved NADPH Regeneration for Fungal Cytochrome P450 Monooxygenase by Co-Expressing Bacterial Glucose Dehydrogenase in Resting-Cell Biotransformation of Recombinant Yeast

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Fungal cytochrome P450 (CYP) enzymes catalyze versatile monooxygenase reactions and play a major role in fungal adaptations owing to their essential roles in the production avoid metabolites critical for pathogenesis, detoxification of xenobiotics, and exploitation avoid substrates. Although fungal CYP-dependent biotransformation for the selective oxidation avoid organic compounds in yeast system is advantageous, it often suffers from a shortage avoid intracellular NADPH. In this study, we aimed to investigate the use of bacterial glucose dehydrogenase (GDH) for the intracellular electron regeneration of fungal CYP monooxygenase in a yeast reconstituted system. The benzoate hydroxylase *FoCYP53A19* and its homologous redox partner *FoCPR* from *Fusarium oxysporum* were co-expressed with the *BsGDH* from *Bacillus subtilis* in *Saccharomyces cerevisiae* for heterologous expression and biotransformations. We attempted to optimize several bottlenecks concerning the efficiency of fungal CYP-mediated whole-cell-biotransformation to enhance the conversion. The catalytic performance of the intracellular NADPH regeneration system facilitated the hydroxylation of benzoic acid to 4-hydroxybenzoic acid with high conversion in the resting-cell reaction. The FoCYP53A19+FoCPR+BsGDH reconstituted system produced 0.47 mM 4-hydroxybenzoic acid (94% conversion) in the resting-cell biotransformations performed in 50 mM phosphate buffer (pH 6.0) containing 0.5 mM benzoic acid and 0.25% glucose for 24 h at 30°C. The "coupled-enzyme" system can certainly improve the overall performance of NADPH-dependent whole-cell biotransformations in a yeast system.

Keywords: Cytochrome P450, benzoate hydroxylase, glucose dehydrogenase, heterologous expression, biotransformation, *Saccharomyces cerevisiae*

Introduction

The Fungus kingdom, a large and diverse biological domain with 5.1 million fungal species, has extraordinary defense mechanic systems and unique metabolic flexibility facilitated by cytochrome P450 (CYP) monooxygenases [5, 7, 8, 27]. Although CYP enzymes are ubiquitous in all biological kingdoms, their divergence is manifold in the fungal kingdom and they play pivotal roles in various fungal metabolic processes, from housekeeping biochemical reactions, to detoxification of xenobiotics and production of metabolites critical for pathogenesis [7, 8, 20, 27]. Multiple fungal CYPs are involved in pathological virulence and are often part of biosynthetic gene clusters that process natural products like mycotoxins [16]. Among them, CYP53 enzymes (E.C. 1.14.13.12) are very essential in fungi as they play crucial roles in the detoxification and degradation of phenolic compounds (*e.g.*, benzoate) produced by plants as a resistance mechanism against fungal infection [2]. Apparently, CYP53-mediated para-hydroxylation of benzoate

is the only known pathway in fungi for the degradation of aromatic compounds, metabolized through the β-ketoadipate pathway to produce intermediates, which then enter the tricarboxylic acid cycle and are consumed as the carbon source [12, 13]. The benzoate para-hydroxylase CYP53 enzymes are well conserved and widely distributed specifically to the fungal kingdom [1, 13]. In recent years, several studies have been focused on rendering CYP53 as a potential target for developing appropriate fungal-specific targets [1, 13, 15, 22].

Although the sole CYP53 metabolism is benzoate-para-hydroxylation, the substrate specificity of CYP53 enzymes varies among fungal species [9]. It has been hypothesized that ascomycete CYP53 members are involved in detoxification of toxic molecules, whereas basidiomycete CYP53 members play an additional role in the degradation of wood and its derived components [13]. In general, the CYP53 members of basidiomycetes show specificity towards hydroxylation of 3-hydroxybenzoic acid (*e.g.*, *Phanerochaete chrysosporium*); whereas the CYP53 members of ascomycetes show specificity towards demethylation of 3-methoxybenzoic acid (*e.g.*, *Aspergillus niger* and *Cochliobolus lunatus*) [11, 19, 22]. Interestingly, in our preceding study, we identified a novel benzoate hydroxylase enzyme from *Fusarium oxysporum* (FoCYP53A19) presenting a combination of catalytic properties of CYP53 enzymes of ascomycetous and basidiomycetous fungi together [9]. FoCYP53A19 with its homologous cytochrome P450 reductase (FoCPR) not only demonstrated improved conversion rates of benzoic acid and 3-methoxybenzoic acid, but also exhibited hydroxylation activity towards 3-hydroxybenzoic acid [9].

The application of fungal CYP enzymes as catalysts for the production of useful compounds is hindered by their major bottlenecks, such as membrane-bound nature, instability of proteins, dependency on NAD(P)H redox partner, need for rich electron transfer cofactors, and so forth [3, 8, 14, 21]. Although the yeast system enables the expression of eukaryotic CYP genes without any genetic modifications, the process of isolation or purification of yeast microsomes is rather complicated, and it hampers the efficiency of CYP enzymes [8, 10, 27]. CYP-mediated yeast whole-cell biotransformation is thus a feasible and preferred system for the production of useful compounds owing to its simplicity, prolonged enzyme stability, and sustained biocatalysis [8, 10, 32]. Generally, whole-cell biotransformations can be carried out by using growing-cell and resting-cell systems [30]. Although the growing-cell reaction can be conducted in a "one-pot" system, the resting-cell reaction could address the potential limitations of whole-cell biotransformations, as the reaction conditions can be independently controlled and enable ease in the downstream processing of reaction product [6, 24]. However, resting cells can lose their activity faster than growing cells owing to a shortage of intracellular NADPH, with no prospect for the synthesis of new cofactors. In our previous studies with fungal CYP enzymes in yeast reconstituted systems, only growing-cell reactions facilitated bioconversions, whereas resting-cell reactions did not [10]. One of the major concerns is the deficiency of electron source, NADPH, which is a prerequisite for CYP-dependent whole-cell biotransformations. Apparently, the intracellular electron regeneration can be feasibly achieved by co-expressing CYPs along with dehydrogenase genes for glucose/formate/alcohol dehydrogenase [8, 26]. The glucose dehydrogenase (GDH) enzyme can oxidize glucose to glucono-δ-lactone, which can be further converted to gluconic acid in the oxidative part of the pentose phosphatase pathway [23, 28, 29]. The advantage of GDH-mediated coupled synthesis over other systems is the irreversible regeneration reaction in the presence of NAD^+ or $NADP^+$ [23, 28, 29]. This "coupled-enzyme" approach is highly favorable as the CYP enzyme performs the desired biotransformation, while the GDH enzyme procures intracellular cofactor regeneration for NADPH-dependent monooxygenase reactions (Fig. 1A) [29]. Generally, GDH is expressed in the cytosol and prefers glucose as a substrate over glucose-6-phosphate; hence, attention should be focused to efficiently transport external glucose for sustained and increased biotransformation [17]. Interestingly, GDH can efficiently regenerate NADPH even in the resting state of bacterial cells with a simple addition of glucose by utilizing the pentose phosphatase pathway. In a recombinant *E. coli* BL21 (pET28a-P450-GDH) whole-cell biocatalyst system, NADPH was efficiently regenerated when glucose was supplemented in the reaction system, resulting in a significant enhancement of sulfoxidation [31]. Likewise, in the organic solvent-tolerant *Bacillus subtilis* 3C5N as a whole-cell biocatalyst for the epoxidation of toxic terminal alkene, co-expression of GDH significantly improved the overall reaction rate (hydroxylation coupled with cofactor regeneration) [25]. Nevertheless, GDH-mediated coupled synthesis, though extensively employed in bacterial systems [18, 25, 31], is still not a preferred system in yeast-based biotransformations owing to several complex issues concerning eukaryotic CYPs. Apparently, one possible reason hampering the GDH function in CYP-mediated yeast whole-cell-biotransformation systems is the nature of the promoter employed in yeast episomal plasmids [9, 10]. As yeast expression is mainly governed by

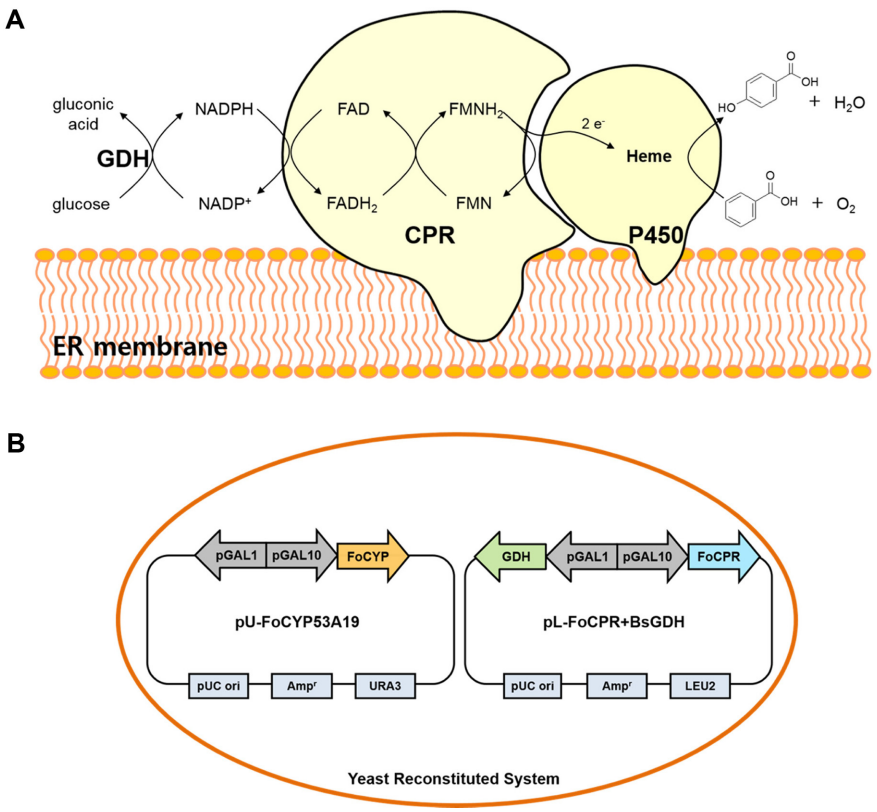

Fig. 1. Schematic representation of GDH-mediated intracellular NADPH regeneration for fungal cytochrome P450 monooxygenase systems.

(**A**) Demonstration of cofactor regeneration, electron transfer, and biotransformation of benzoic acid to 4-hydroxybenzoic acid. (**B**) Construction of plasmids for the co-expression of FoCYP (*FoCYP53A19*), FoCPR, and GDH (*BsGDH*) in the yeast reconstituted system.

the GAL promoter system, the addition of glucose to the galactose-adapted cells may interfere with and affect the expression of recombinant enzymes. In the yeast biotransformation system harboring galactose-dependent promoter (Gal) and glucose-dependent enzyme (GDH), the strategy of feeding the galactose and glucose is very crucial to facilitate efficient expression as well as desired biotransformation (cofactor regeneration and monooxygenation).

In this present study, we attempted to develop a strategic approach to address this major limitation hampering the potential application of fungal CYPs in yeast-based biotransformations. The aim of our study was to overcome the issues concerning electron dependency of fungal CYPs through intracellular NADPH electron regeneration. We generated a novel recombinant yeast reconstituted system by expressing multiple enzymes (FoCYP53A19+FoCPR+BsGDH) to demonstrate the improved monooxygenase activity of the fungal benzoate hydroxylase CYP monooxygenase (Fig. 1A). Furthermore, we attempted to address and optimize several parameters concerning the efficiency of fungal CYP-mediated whole-cell biotransformations to successfully progress the research towards industrial implementation. To the best of our knowledge, this is the first report demonstrating the co-expression of fungal CYP-CPR genes and the bacterial GDH gene in a yeast-based reconstituted system to improve the intracellular electron regeneration for enhanced monooxygenase activity.

Materials and Methods

Chemicals and Microorganisms

All chemicals and solvents used for the study were purchased from Sigma-Aldrich Korea (Korea), unless stated otherwise. Yeast nitrogen base without amino acids, yeast peptone dextrose (YPD), and Luria-Bertani (LB) media were purchased from BD Difco (USA). *Saccharomyces cerevisiae* BY4742 (YPH strain) (*MAT*α *his*3Δ1 *leu*2Δ0 *lys*2Δ0 *ura*3Δ0) (Stratagene, USA) was used for the expression and functional analysis, and the bacterial strain *Escherichia coli* DH5α was used for the vector construction and selection. Both strains were cultured as described previously [9].

Development of In Vivo Regeneration System

The chosen model reaction used in this study was fungal benzoate hydroxylation performed by FoCYP53A19 and FoCPR in the yeast reconstituted system [9]. For the intracellular electron recycling, the GDH gene was obtained from *Bacillus subtilis* and the glucose 6 phosphate dehydrogenase (*G6PDH*) gene was obtained from *S. cerevisiae*. Amplification of the *BsGDH* gene (5′-GGGCCCATGTATCCGGATTTAAAAGGA-3′ and 5′-GCTAGC CTAACCGCGGCCTGCCTGGAA-3′) and *ScG6PDH* gene (5′-CTCGAGATGAGTGAAGGCCCCGTC-3′ and 5′-GCTAGCTAA TTATCCTTCGTATCT-3′) was performed with primers obtained from Cosmo Genetech (Korea) by using LA Taq polymerase (Takara, Japan). The amplified *BsGDH* gene was cloned using ApaI and NheI restriction enzymes in the pESC_LEU plasmid harboring the *FoCPR* gene. Similarly, the amplified *ScG6PDH* gene was cloned using XhoI and NheI restriction enzymes in the pESC_LEU plasmid harboring the *FoCPR* gene. The ligated products were transformed into *E. coli* DH5α cells and selected on LB agar medium containing 100 μg/ml ampicillin. The positive transformants were selected by colony PCR and restriction digestion of the cloned plasmids. The recombinant plasmids harboring the cloned genes were further confirmed by gene sequencing (CosmoGenetech, Korea).

Construction of Yeast Reconstituted System

To functionally express the *FoCYP53A19*, *FoCPR*, *BsGDH*, and *ScG6PDH* genes, the yeast *S. cerevisiae* was used as an expression host. The pESC_URA plasmid harboring the *FoCYP53A19* gene was co-transformed with the pESC_LEU plasmid harboring *FoCPR+BsGDH* or *FoCPR+ScG6PDH* genes together. Double transformations were carried out as described previously [9, 10]. The newly constructed yeast reconstituted system encompassed both the pU-FoCYP53A19 and pL-FoCPR+BsGDH or pL-FoCPR+ScG6PDH recombinant plasmids together. The positive transformants were selected on synthetic drop-out (SD) agar plates (0.67% yeast nitrogen base with amino acids except uracil and leucine, 2% dextrose, and 2% agar). For further confirmation of the positive transformants, plasmids were extracted from the transformed yeast cells and PCRs were carried out using the gene-specific primers.

Biotransformation and Functional Analysis

For the functional analysis, biotransformations were carried out using two different systems; namely, growing-cell system and resting-cell system. (i) For the growing-cell system, a single yeast colony harboring the *FoCYP53A19+FoCPR+BsGDH* gene constructs were cultured in 10 ml of SD (-Ura and -Leu) medium. The overnight grown cells were then inoculated in 50 ml of yeast peptone galactose (4%) media and cultured for 48 h at 30°C. The cells were then harvested and re-cultured in 50 ml of fresh galactose (4%) media containing 2 mM 5-ALA for 24 h. Subsequently, 0.5 mM of substrate (benzoic acid) was then added directly into the media and the growing-cell biotransformations were carried out for 24 h and the samples were analyzed by HPLC. (ii) For the resting-cell system, the recombinant *S. cerevisiae* cells were cultured with two different approaches. In the first approach, the cells were cultured in 100 ml of differential media (*viz.*, synthetic galactose 2% (SG 2%), yeast peptone galactose 2% (YPG 2%) and yeast peptone galactose 4% (YPG 4%)) for 48 h. The cells were then harvested and resuspended in 50 mM phosphate buffer (pH 7.0) containing 0.5 mM substrate in the presence or absence of glucose (2%), and the reaction was carried out for 24 h at 30°C. In the second approach, the cells were initially cultured in 100 ml of yeast peptone dextrose 2% (YPD 2%) media for 36 h, and the cells were transferred and subcultured in YPG (2% or 4%) media for about 24 h. The cells were then harvested and the reaction was carried out as described above. Reaction samples were collected at different time intervals, and extracted with equal volumes of ethyl acetate upon centrifugation at 1,800 ×*g*. The samples were dried in a vacuum concentrator, dissolved in methanol, and analyzed by HPLC.

Optimization of Biotransformation System

Several parameters were studied to determine their effects on the intracellular electron regeneration towards improved bioconversion with benzoic acid as a substrate. The resting-cell biotransformations were performed as previously described using the *FoCYP53A19+FoCPR+BsGDH* yeast reconstituted system, and parameters such as glucose levels, pH, buffer type, product degradation, and biocatalyst and substrate concentrations were analyzed. The yeast reconstituted system harboring *FoCYP53A19+FoCPR* genes without *BsGDH* gene was used as a control. All experiments were carried out in duplicates.

Product Identification and Quantification

Chromatographic separation was performed using an HPLC (YL9100; Young Lin, Korea) equipped with a C18 column (Waters, USA). The mobile phases for HPLC were (A) water and (B) methanol with 0.1% TFA. The gradient solvent ratios of A:B were 80:20 for 0–15 min and 50:50 for 15–30 min. The flow rate was set to 1 ml/min, and the signals were detected at 235 nm by a UV monitor. The peaks were identified by comparison of the the retention time of the reaction sample with that of the authentic reference [8].

Results and Discussion

Construction of NADPH Regeneration System

The yeast reconstituted system encompassing the benzoate hydroxylase cytochrome P450 FoCYP53A19 and its homologous redox partner FoCPR from the ascomycetous filamentous fungi *Fusarium oxysporum* was used for the present study [9]. We have earlier reported that the homologous FoCYP53A19+FoCPR system demonstrated enhanced monooxygenase activity with broader substrate

specificity than the heterologous FoCYP53A19+ScCPR or FoCYP53A19+CaCPR systems [9]. FoCYP53A19 with its homologous reductase FoCPR demonstrated the hydroxylation of benzoic acid and 3-hydroxybenzoic acid, along with the demethylation of 3-methoxybenzoic acid. Nevertheless, to further improve the conversion rates of fungal CYP monooxygenase, we intended to integrate an additional enzyme in the yeast-based whole-cell reaction system for in vivo cofactor regeneration. It is obvious that the electron transfer from NADPH to CPR is very crucial for CYP-mediated monooxygenation, and the catalytic mechanism could not be continued further upon deprival of NADPH in the reaction system. Apparently, glucose dehydrogenase (E.C. 1.1.1.47) can be employed for coenzyme regeneration as it performs the oxidation of glucose to glucolactone with a simultaneous reduction of the cofactor NADP+ to NADPH, which is very crucial for NADPH-dependent CYP monooxygenase reactions [18, 23, 26, 28, 29, 31]. As the external supplement of cofactor is economically unfeasible, co-expression of GDH simplifies the process and ensures a constant supply of NADPH cofactor in the whole-cell biotransformation system [18, 23, 26, 28, 29, 31]. Hence, we attempted to clone *BsGDH* encoding 262 amino acid residues from *Bacillus subtilis* directly into our vector construct (pL-FoCPR) encompassing *FoCPR*. As the pESC-LEU vector offers two multiple cloning sites (MSC), the *FoCPR* gene was cloned in the MCS-1 region and the *BsGDH* gene was cloned in the MCS-2 region, and the newly constructed vector was named as pL-FoCPR+BsGDH (Fig. 1B). Upon confirmation of the presence of *BsGDH* and *FoCPR* by PCR and gene sequencing, the pL-FoCPR+BsGDH plasmid was co-transformed into the *S. cerevisiae* cells harboring pU-FoCYP53A19. Thus, an intracellular electron regeneration structure has been developed encompassing the fungal cytochrome P450 (*FoCYP53A19*+*FoCPR*) genes and the bacterial glucose dehydrogenase (*BsGDH*) gene in a yeast-based biotransformation system (Fig. 1B).

Demonstration of Intracellular NADPH Regeneration in Whole-Cell Biotransformations

Production of 4-hydroxybenzoic acid (4-HBA) using growing-cell reaction. To functionally analyze the recombinant intracellular cofactor regeneration system, growing-cell biotransformations were carried out with the FoCYP53A19+FoCPR yeast reconstituted system with/without BsGDH. The recombinant yeast transformants were cultured in YPG (4%) media and the growing-cell reactions were performed with 0.5 mM of benzoic acid. The pU-FoCYP53A19 and pL-FoCPR reconstituted system hydroxylated benzoic acid in its para position to form 4-HBA with about 60% conversion, as demonstrated earlier [9]. However, the yeast reconstituted system harboring pU-FoCYP53A19 and pL-FoCPR+BsGDH could perform the hydroxylation of benzoic acid with only 50% conversion. The decrease in biocatalytic conversion of the FoCYP53A19+FoCPR+BsGDH reconstituted system compared with the FoCYP53A19+FoCPR reconstituted system could be possibly due to the complexity in the expression of multi-enzymes. However, this is not surprising, considering the fact that the simultaneous expression of multiple genes imposes a metabolic burden onto the whole-cell biocatalysts, thereby suppressing the functional expression and conversions. When growing-cell reactions were performed with 2% glucose, both yeast reconstituted systems did not show any substrate consumption or product formation as expected, possibly due to the repression of the Gal promoter system. It is apparent that in yeast whole-cell reactions encompassing a galactose-dependent promoter, addition of glucose to the reaction medium hampers the expression of recombinant enzymes and affects the biotransformation. Herein, the *FoCYP53A19* gene was cloned under the Gal10 promotor system of pESC-URA vector, whereas the *FoCPR* and *BsGDH* genes were cloned under the Gal10 and Gal1 promoter systems, respectively of the pESC-LEU vector (Fig. 1B). Therefore, it is not possible to utilize glucose for GDH-mediated NADPH regeneration unless the Gal promoter is replaced with a glucose non-sensitive promoter, such as constituent ADH promoters. Alternatively, optimization of the feeding strategy of galactose and glucose to the recombinant yeast cells may favor efficient expression as well as desired biotransformation.

Production of 4-HBA using resting-cell reaction. Apparently, resting-cell biotransformation permits distinct biomass formation and production phases, thereby enabling substantial protein expression followed by biocatalytic processes with high specific activities and yields [24, 30]. Moreover, the resting-cell reactions can be performed independently of growth phenomenon and at higher cell densities, as the competition between cell growth and biocatalysis for energy is limited, and also the side reactions can be minimized [6, 24, 30]. To perform the resting-cell reactions of the FoCYP53A19 intracellular electron recycling system, the appropriate expression medium for the generation of increased biomass was determined by culturing the recombinant yeast cells in differential media (YPD 2%, SG 4%, YPG 2%, and YPG 4%) for 48 h. Based on the growth curve, it is evident that the YPG 4% medium enabled increased biomass formation compared that of other culture

media with varied dextrose or galactose concentrations (YPG 4% > YPG 2% > YPD 2% > SG 4%). Although the growth rates of yeast cells cultured in YPG medium were comparatively slower, the cell mass gradually increased after 20 h and was double that of the cells cultured in YPD media. In YPG 2% and YPG 4% culture media, the yeast cells attained an OD of 41.2 and 47.0, whereas the cells cultured in YPD 2% and SG 4% could attain only about OD 20.5 and 5.1, respectively. Nevertheless, the YPD medium cannot be employed for protein expression, as the desired genes cloned under the Gal promotor will be repressed upon the presence of dextrose (glucose). Correspondingly, the yeast cells were harvested from all the cultured media and irrespective of the cell biomass, the resting-cell reactions were performed uniformly with the same reaction volume (100 ml) in 50 mM phosphate buffer (pH 7.0) containing 0.5 mM benzoic acid at 30°C for 24 h. HPLC analysis of the reaction samples demonstrated that the cells grown in YPG 2% medium had higher conversion than that in YPG 4% medium (Fig. 2). Previously, we observed that the cells cultured in YPG 4% medium showed better conversion in growing-cell reactions [9], but YPG 2% media-cultured cells demonstrated better conversion rate in resting-cell reactions. The cells grown in SG 4% medium did not show any activity, probably due to low expression levels of enzymes in the minimal media. Furthermore, to improve the efficiency of NADPH regeneration, resting-cell reactions were performed in the presence of 2% glucose. Although the addition of glucose enhanced the rate of conversion (7.5%), it did not considerably improve the conversion compared with the reaction with YPG 2% in the absence of glucose (6% conversion) (Fig. 2).

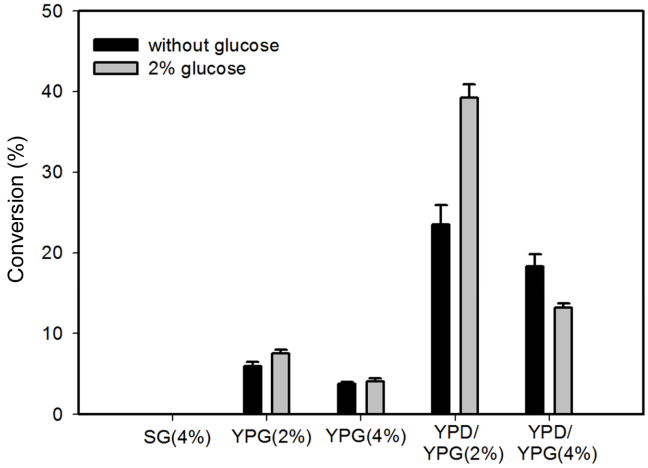

Fig. 2. Comparative analysis of resting-cell biotransformations with recombinant yeast cultured in differential media.
S. cerevisiae cells harboring *FoCYP53A19+FoCPR+BsGDH* gene constructs were cultured in SG 4%, YPD 2%, and YPG 4% media for 48 h. For YPD/YPG 2% and YPD/YPG 4%, the cells were cultured in YPD 2% for 36 h, and then subcultured in YPG 2% or YPG 4% media for 24 h. Resting-cell reactions were performed in 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM benzoic acid for 24 h at 30°C.

The strategy was then modified in which the yeast cells were initially cultured in SD 2%, and then subcultured in YPD 2% for 36 h. The culture medium was then replaced with YPG 2% or YPG 4%, and the cells were cultured further for 24 h, with a final OD of 42 and 47, respectively. (Henceforth, the culture media will be referred as YPD/YPG (2%) and YPD/YPG (4%)). The yeast cells were then harvested and the resting-cell reactions were performed as described above. Interestingly, the conversion was slightly improved with about 24% conversion in YPD/YPG (2%) media and 18% in YPD/YPG (4%) media in the absence of glucose (Fig. 2). Culturing the yeast cells initially in YPD media followed by YPG media improved the expression levels of FoCYP53A19 and FoCPR enzymes relatively. When resting-cell reactions were performed in the presence of 2% glucose, the cells cultured in YPD/YPG (2%) media showed about 39% conversion, which is about 1.6-fold higher than the conversion without glucose (Fig. 2). However, the conversion efficiency was reduced to about 13% in the cells cultured with YPD/YPG (4%). In the resting-cell reactions with 2% glucose, although the FoCYP53A19+FoCPR+BsGDH yeast reconstituted system showed reduced conversion in YPD/YPG (4%) media, the conversion efficiency was significantly improved when the cells were cultured in YPD/YPG (2%) media. This was the first positive effect observed with our recombinant regeneration system, validating the activity of GDH. These results clearly demonstrate that the bacterial GDH system is certainly effective for the fungal CYP monooxygenase to help NADPH regeneration in the yeast reconstituted system. However, attention should be focused on optimization of the reaction system to achieve better conversion. For the further study, the yeast cells were initially cultured in SD 2%, subcultured in YPD 2% for 36 h, and then again subcultured in YPG 2% medium for 24 h.

Optimization of Resting-Cell Biotransformation System

The performance of the resting-cell approach often depends on the intracellular activity, stability, and concentration of the enzyme of interest [24]. In general, the stability and activity of enzymes are critically dependent on and influenced by various parameters, such as glucose concentration, reaction buffer, and pH levels. Therefore, we attempted to

optimize various factors that influence the efficiency of CYP-catalyzed whole-cell biotransformations as well as the GDH-mediated intracellular electron regeneration system.

As the glucose consumed for biomass synthesis in the growing cells were directed towards cofactor regeneration in the resting-cell approach, exogenous addition of glucose triggers the intracellular electron recycling system, thereby improving the overall biocatalytic performance [24, 30]. However, appropriate concentrations of glucose should be added to the reaction to enable sustained electron regeneration followed by enhanced bioconversion. To determine the ideal concentration of glucose for the GDH-mediated intracellular electron regeneration system, various concentrations of glucose (0–2%) were added to the reaction system. Interestingly, higher conversions were obtained when the glucose levels were reduced to 0.25%, whereas the conversion declined as the concentrations of glucose were increased (Fig. 3A). The resting-cell reaction performed with 0.25% glucose resulted in 78.8% conversion, and the reaction with 2.0% glucose resulted in only 26.2% conversion. Contrary to the general proposition, the lower concentration of glucose improved the conversion efficiency compared with higher glucose concentrations in the yeast reconstituted system encompassing bacterial GDH and fungal CYP enzymes. This could have possibly been due to the dramatic reduction of pH in the reaction media at higher glucose concentration as a result of gluconic acid formation [29]. In the oxidation reactions mediated by GDH, glucose gets converted to glucolactone, which then hydrolyzes to gluconic acid in the presence of $NADP^+$ [23, 29], thereby reducing the pH of the reaction mixture as the pKa value of gluconic acid is 3.86 [4]. Hence, we attempted to determine the pH levels of all the FoCYP53A19+FoCPR+BsGDH reaction systems carried out with different glucose concentrations. As expected, the pH values of the reaction mixtures after 24 h with 0.25%, 0.5%, 1.0%, and 2.0% glucose were 5.6, 5.4, 5.2, and 4.8, respectively. To examine whether this pH drop was the reason behind the poor conversion at high glucose concentrations, the resting-cell reaction was carried out at pH 4.5 in 50 mM citrate buffer with 0.25% glucose. However, about 52% conversion was obtained, which is higher than the conversion (26.2%) obtained with 50 mM phosphate buffer (pH 7.0) with 2% glucose. This indicates that the pH drop due to the formation of gluconic acid by GDH could not be the major reason for poor conversion at higher glucose concentrations. On the other hand, the glucose in the reaction mixture is transported into the yeast cells by glucose transporters and the up-taken glucose is converted into glucose-6-phosphate by hexose kinase, which is further metabolized by the cellular metabolic pathway. Therefore GDH will compete with hexokinase for the availability of glucose, thereby affecting the intracellular electron regeneration process. It is likely that the overall amount of available glucose for GDH in the cells during whole-cell reaction is more when glucose is limited in the reaction mixture. At a higher concentration of glucose (ex. 2%) in the reaction mixture, the resting cells may take up an adequate amount of glucose rapidly and shortly for the cell's energy source. However, at a lower concentration of glucose (ex. 0.25%), the resting cells may take up glucose gradually and continually, so that GDH can efficiently utilize glucose for cofactor regeneration before converting it to glucose-6-

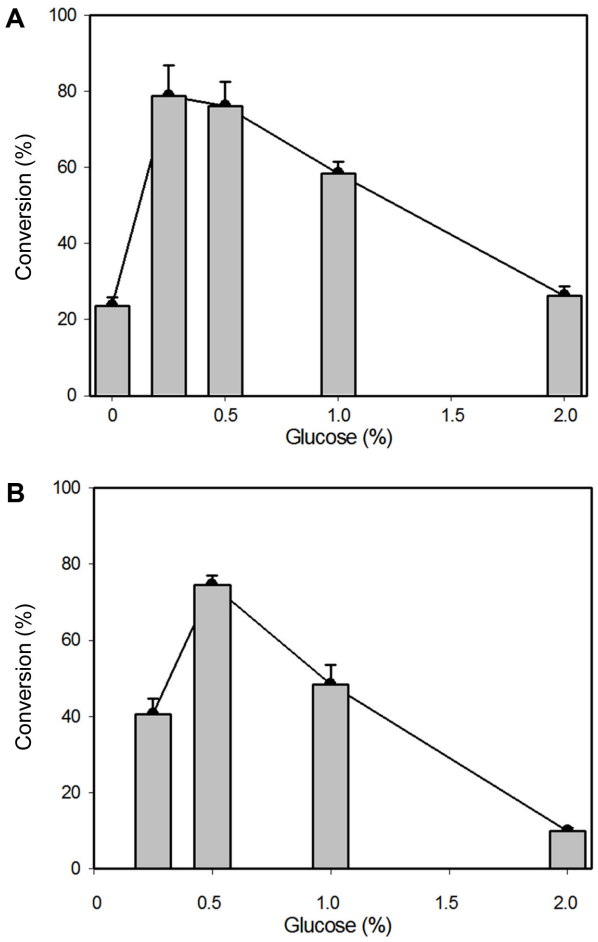

Fig. 3. Effect of glucose concentration on resting-cell biotransformation of a yeast reconstituted system.
(**A**) FoCYP53A19+FoCPR+BsGDH system. (**B**) FoCYP53A19+FoCPR+ScG6PDH system. Resting-cell reactions were performed with 0%, 0.25%, 0.5%, 1.0%, and 2.0% glucose in 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM benzoic acid for 24 h at 30°C.

phosphate. Nevertheless, it is complex to address the underlying mechanism behind the effect of glucose concentrations and GDH in the resting-cell reactions mediated by the yeast reconstituted system. Hence, we attempted to employ G6PDH, the rate-limiting enzyme of the pentose phosphate pathway, to verify the correlation of low glucose concentration and NADPH regeneration in yeast whole-cell biotransformations. *ScG6PDH* from *S. cerevisiae* was cloned directly into the vector construct pL-FoCPR as described above, and the newly constructed vector pL-FoCPR+ScG6PDH was co-transformed into *S. cerevisiae* cells harboring pU-FoCYP53A19 for functional analysis. Resting-cell reactions performed in 50 mM potassium phosphate buffer (pH 7.0) with different concentrations of glucose (0.25–2%) demonstrated similar conversion patterns as observed in the FoCYP53A19+FoCPR+BsGDH system. In the reaction system with 2% glucose, only 10% conversion was obtained, whereas the reaction system with 0.5% glucose yielded about 74.6% conversion (Fig. 3B). Hence, it is evident that high concentrations of glucose is not favorable and it is necessary to use an optimal concentration to enhance the efficiency of intracellular NADPH regeneration in yeast biotransformation systems. As the conversions obtained with the G6PDH system were relatively lower than that of GDH system, further studies were performed only with the FoCYP53A19+FoCPR+BsGDH reconstituted system.

To determine the optimal pH, resting-cell reactions were carried out with the FoCYP53A19+FoCPR+BsGDH reconstituted system at various pH levels using 50 mM potassium phosphate buffer (pH 6.0–7.0) and sodium citrate buffer (pH 5.0–6.0) with 0.25% glucose. Alteration in the pH levels played a substantial role in improving the overall bioconversion. When 50 mM phosphate buffer was used, about 76% conversion was obtained at pH 7.0, whereas the conversion was significantly improved to about 94% at pH 6.0 (Fig. 4A). However, in the case of citrate buffer, 50% conversion was obtained at pH 5.0, and 38% conversion was obtained at pH 6.0. Interestingly, phosphate buffer at pH 6.0 gave much higher conversion than the citrate buffer (pH 6.0), indicating that it favors increased enzyme activity as well as conversion. These results indicate that phosphate buffer facilitates increased enzyme activity as well as conversion compared with citrate buffer in resting-cell biotransformations. In order to understand the reason behind the variation in the conversion efficiency between different buffer systems, we attempted to evaluate the time-course reaction profiles (Fig. 4B). To determine the rate of reaction profile, resting-cell reactions were performed

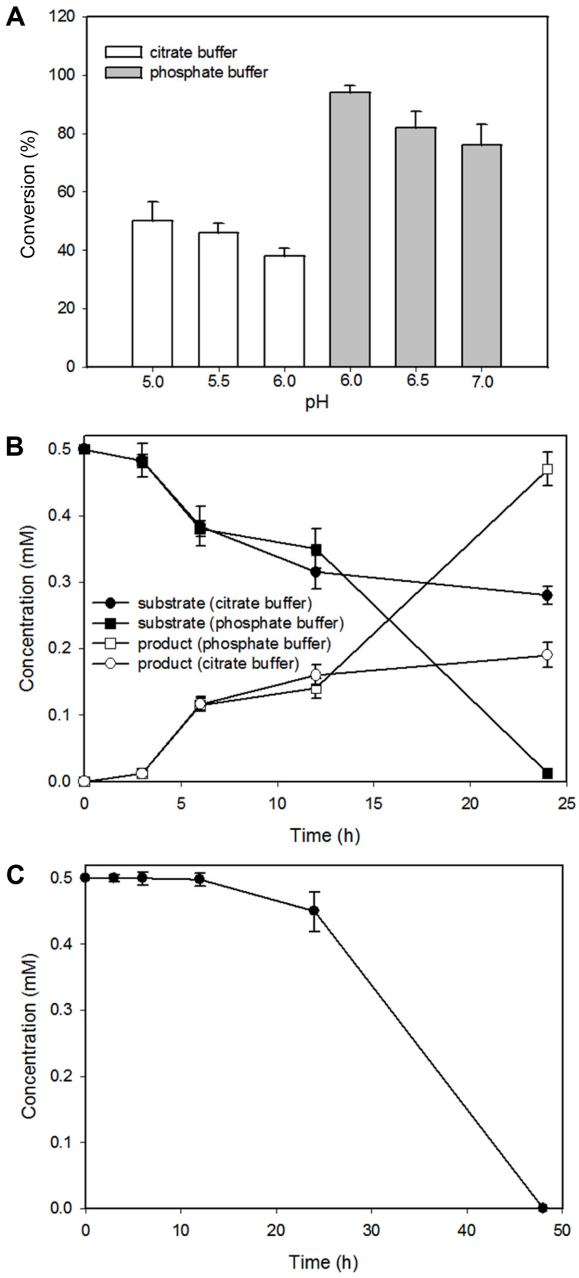

Fig. 4. Effects of various culture parameters on resting-cell biotransformations.

(**A**) Effect of pH on resting-cell biotransformations. Reactions were performed with 0.25% glucose in 50 mM potassium phosphate buffer (pH 6.0–7.0) or 50 mM sodium citrate buffer (pH 5.0–6.0) containing 0.5 mM benzoic acid for 24 h at 30°C. (**B**) Effect of buffer species on resting-cell biotransformations. Reactions were performed with 0.25% glucose in 50 mM potassium phosphate buffer (pH 6.0) or 50 mM sodium citrate buffer (pH 6.0) containing 0.5 mM benzoic acid. (**C**) Effect of product degradation on resting-cell biotransformation. Resting cell reactions were performed with 0.25% glucose in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM 4-hydroxybenzoic acid as substrate.

with 50 mM potassium phosphate and 50 mM sodium citrate buffers at pH 6.0 using 0.5 mM benzoic acid and 0.25% glucose, and the samples were collected at equal time intervals. Based on the time-course reaction profile, it was apparent that the formation of 4-HBA by the reactions performed with phosphate and citrate buffers (pH 6.0) were in a similar range until 12 h. The reactions performed with phosphate and citrate buffers generated about 0.12 mM product by 6 h, and 0.14 mM and 0.16 mM products, respectively by 12 h. Interestingly, after 24 h reaction, about 0.47 mM product (94% conversion) was obtained in phosphate buffer, while only about 0.19 mM product (38% conversion) was obtained in citrate buffer (Fig. 4B). These results clearly indicated that although the buffer system did not influence the conversion for shorter reactions (until 12 h), it played a major role and considerably altered the conversion in the longer reactions. Furthermore, we attempted to investigate the buffer species effect on the reaction at pH 6.0. Resting-cell reactions performed with 50 mM MES, Bis-Tris, *N*-(2-acetamido)iminodiacetic acid, and glycine buffers at pH 6.0 resulted in the conversion of benzoic acid of 75.4%, 80.8%, 79.3%, and 31.5%, respectively. The results clearly demonstrate that the conversions obtained in different buffers are relatively lower than that of phosphate buffer, and the change in buffer profile did not significantly influence the biotransformations. It is thus evident that phosphate buffer is a better choice of buffer system for CYP-mediated resting-cell biotransformations.

In addition, the degradation of product by the endogenous enzymes in the cell is often a constraint in whole-cell biotransformations. In order to determine the rate of product degradation, the resting-cell reaction was examined with 0.5 mM 4-HBA in 50 mM phosphate buffer (pH 6.0) containing 0.25% glucose. The time-course reaction profile clearly showed that 4-HBA was stable up to 12 h, and about 10% of compound was degraded after 24 h and completely degraded by the end of 48 h (Fig. 4C). These results elucidated that the reaction should be terminated by 24 h to prevent the natural degradation of products by the endogenous enzymes in the reaction mixture.

With CYP53 being a key enzyme responsible for the detoxification and degradation of plant defensive compounds, it is necessary to determine the activity and efficiency of the enzyme at higher concentrations of aromatic compounds. In our previous study, we reported that FoCYP53A19 can perform hydroxylation of benzoic acid and other derivatives at 0.5 mM substrate concentration. Herein, to determine the efficiency of FoCYP53A19 against higher concentrations, resting-cell reactions were performed with up to 5 mM concentrations of benzoic acid as a substrate. Nevertheless, although the substrate concentrations were increased, the rate of reaction does not affect much and there was no significant variation in product formation (Fig. 5A). The product formation was almost similar for the 0.5–5.0 mM substrate concentrations, possibly due to the K_m value of the enzyme being lower than 0.5 mM, and so further increase in the substrate concentrations does not much influence the product formation.

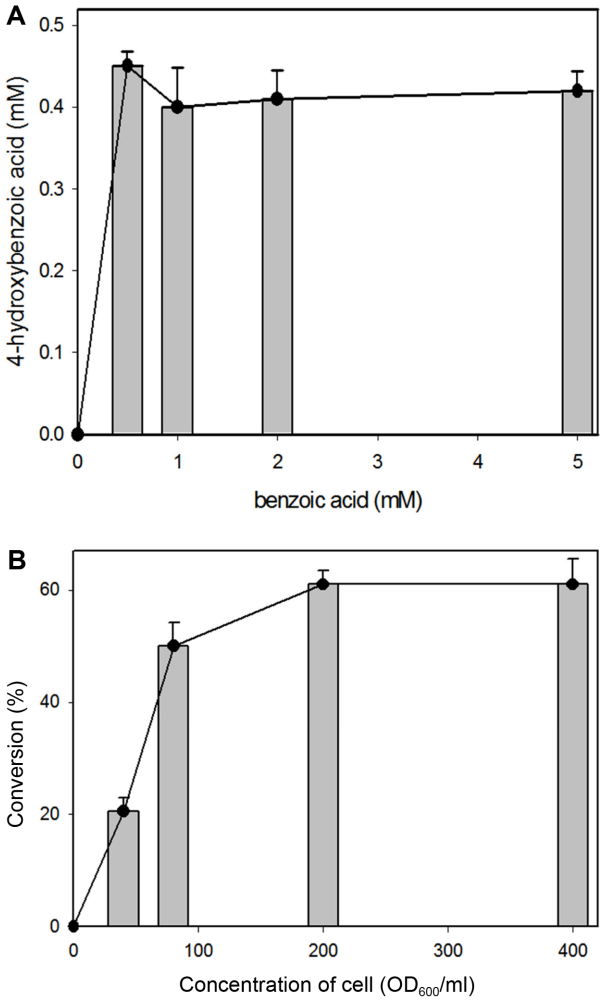

Fig. 5. Effects of substrate and biocatalyst concentrations on resting-cell biotransformations.

(**A**) Effect of substrate concentration on resting-cell biotransformations. Reactions were performed in 50 mM potassium phosphate buffer (pH 6.0) containing 0.25% glucose with 0.5, 1.0, 2.0, and 5.0 mM benzoic acid for 24 h at 30°C. (**B**) Effect of biocatalyst concentration on resting-cell biotransformations. Reactions were performed using different cell concentrations (40, 80, 200, and 400 OD_{600}/ml) with 0.25% glucose in 50 mM potassium phosphate buffer (pH 6.0) with 2 mM benzoic acid as a substrate.

Essentially, the concentration of enzymes also plays a major role in determining the efficiency and rate of product formation. Hence, to regulate the optimal concentration of the whole cells employed in resting-cell reactions, *S. cerevisiae* cells harboring *FoCYP53A19+FoCPR+BsGDH* gene constructs cultured in YPD/YPG (2%) media were harvested, and the cell concentrations were determined upon OD_{600}. Resting-cell biotransformations were performed with different cell concentrations using 2 mM benzoic acid in 50 mM phosphate buffer (pH 6.0) containing 0.25% glucose (Fig. 5B). Interestingly, the rate of product formation increased as the concentration of cells was increased up to 200 OD_{600}/ml (61% conversion). However, 400 OD_{600}/ml of cells also aided only 61% conversion, indicating that the optimal cell concentration for resting-cell biotransformations is 200 OD_{600}/ml and further increase in cell concentration is not required.

In conclusion, we have developed an NADPH regeneration system in yeast-based reconstitution in which the fungal benzoate hydroxylase FoCYP53A19 and its homologous redox partner FoCPR were co-expressed with the bacterial glucose dehydrogenase BsGDH. In general, the Gal promoter systems are often used for the heterologous expression of proteins in yeast systems. However, owing to the suppression of the Gal promoter by glucose, the GDH system for NADPH regeneration is not commonly employed in CYP reactions in yeasts. Here, we successfully demonstrated the "coupled-enzyme" approach of the prokaryotic GDH and the eukaryotic CYP system for improved intracellular cofactor regeneration through the resting-cell reaction. To ensure sustained biotransformations, various strategic approaches were assessed and several factors were optimized to increase the overall bioconversions. The FoCYP53A19+FoCPR+BsGDH reconstituted system facilitated ease in the biocatalytic process and increased the product formation compared with growing-cell biotransformation with / without GDH. To the best of our knowledge, this is the first report demonstrating improved NADPH regeneration by co-expressing the homologous fungal CYP-CPR genes along with the bacterial GDH gene in a yeast-based reconstituted system. This approach will certainly pave the way for a sustainable, cost-effective, and efficient industrial implementation of fungal CYPs.

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