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# UV-dependent production of 25-hydroxyvitamin $D_2$ in the recombinant yeast cells expressing human CYP2R1

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ABSTRACT

CYP2R1 is known to be a physiologically important vitamin D 25-hydroxylase. We have successfully expressed human CYP2R1 in *Saccharomyces cerevisiae* to reveal its enzymatic properties. In this study, we examined production of 25-hydroxylated vitamin D using whole recombinant yeast cells that expressed CYP2R1. When vitamin $D_3$ or vitamin $D_2$ was added to the cell suspension of CYP2R1-expressing yeast cells in a buffer containing glucose and β-cyclodextrin, the vitamins were converted into their 25-hydroxylated products. Next, we irradiated the cell suspension with UVB and incubated at 37 °C. Surprisingly, the 25-hydroxy vitamin $D_2$ was produced without additional vitamin $D_2$. Endogenous ergosterol was likely converted into vitamin $D_2$ by UV irradiation and thermal isomerization, and then the resulting vitamin $D_2$ was converted to 25-hydroxyvitamin $D_2$ by CYP2R1. This novel method for producing 25-hydroxyvitamin $D_2$ without a substrate could be useful for practical purposes.



## 1. Introduction

It is commonly known that vitamin $D_3$ is initially converted to 25(OH)$D_3$ in the liver, and then 25(OH)$D_3$ is converted to a functionally active form, namely 1α,25$(OH)_2D_3$, in the kidney. 1α,25$(OH)_2D_3$ plays essential roles in calcium and phosphate homeostasis, cell differentiation and immunology [1]. The metabolic activation of vitamin $D_3$ is performed by the hepatic vitamin $D_3$ 25-hydroxylases (microsomal CYP2R1 and mitochondrial CYP27A1) and the renal 25(OH)$D_3$ 1α-hydroxylase (mitochondrial CYP27B1). Cheng et al. [2] cloned the gene encoding human CYP2R1, and they succeeded in the functional expression of human CYP2R1 in HEK 293 cells. They detected CYP2R1-dependent 25-hydroxylation activity toward vitamin $D_3$ and vitamin $D_2$. They also reported that the CYP2R1 mutant L99P abolished vitamin D 25-hydroxylase activity and that the CYP2R1 enzyme mutation causes selective 25-hydroxyvitamin $D_3$ deficiency [3]. These data suggest that CYP2R1 is a physiologically important vitamin D 25-hydroxylase. We have successfully expressed CYP2R1 in *Saccharomyces cerevisiae* cells, and examined its enzymatic properties [4]. Kinetic studies using microsomes of the recombinant yeast cells have suggested that CYP2R1 is a more important vitamin $D_3$ 25-hydroxylase than CYP27A1. In addition, we found that CYP2R1 and CYP27A1 were clearly different in the metabolism of vitamin $D_2$. The CYP2R1 hydroxylated vitamin $D_2$ at position C-25 position (Fig. 1) and the CYP27A1 hydroxylated the vitamin at positions C-24 and C-27 [4,5].

Many studies have revealed that a reduction in the serum 25(OH)D level (sum of 25(OH)$D_3$ and 25(OH)$D_2$) is closely linked with osteoporosis, numerous cancers, and chronic diseases [6–8]. Thus, a periodic measurement of the serum 25(OH)D concentration appears to be important in maintaining good health. Currently, serum 25(OH)D concentration is mostly measured with a liquid chromatograph–mass spectrometer (LC–MS). Thus, a demand of 25(OH)$D_3$ and 25(OH)$D_2$ for use as the authentic standards will increase in the near future. Because their chemical synthesis from vitamin $D_3$ or vitamin $D_2$ requires a multi-step and costly synthesis, a single-step bioconversion would be attractive. We attempted to use CYP2R1 to produce both 25(OH)$D_3$ and 25(OH)$D_2$. We have established a novel method to produce 25(OH)$D_2$ by using CYP2R1-expressing yeast cells without additional substrates.

## 2. Materials and methods

### 2.1. Materials

Vitamin $D_3$, vitamin $D_2$, 25(OH)$D_3$, and ergosterol were purchased from Wako Pure Chemicals (Osaka, Japan). 25(OH)$D_2$, 1α, 25$(OH)_2D_3$ and 1α, 25$(OH)_2D_3$ were purchased from Sigma–Al-

*Abbreviations:* 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; 25(OH)$D_2$, 25-hydroxyvitamin $D_2$; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; 1α,25$(OH)_2D_2$, 1α,25-dihydroxyvitamin $D_2$; CYP, cytochrome P450.

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drich. (St. Louis, MO). 1α(OH)$D_3$ was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). 1α(OH)$D_2$ was purchased from Calbiochem (La Jolla, CA, USA). NADPH was purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were of the best commercially available grade.

### 2.2. Cultivation of the recombinant S. cerevisiae cells

The recombinant *S. cerevisiae* cells expressing human CYP2R1 were cultivated in a synthetic minimal medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, and 20 mg/L histidine at 30 °C.

### 2.3. Production of 25-hydroxylated vitamin Ds by the recombinant S. cerevisiae cells expressing human CYP2R1 with substrate

The recombinant *S. cerevisiae* cells expressing human CYP2R1 were cultivated in the medium described above, and pelleted by centrifugation while the cells were in log phase. The cells were washed with 0.1 M potassium phosphate buffer (pH 7.4), at a cell concentration of 1–3 × $10^9$ cells/mL. After centrifugation, the pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 4% glucose and 0.2% 3-hydroxy-β-cyclodextrin. Glucose is needed to regenerate NADPH in *S. cerevisiae* cells. The substrates vitamin $D_3$, vitamin $D_2$, 1α(OH)$D_3$, and 1α(OH)$D_2$ were each dissolved in ethanol and added to the cell suspension at final concentrations of 50 μM (vitamin $D_3$ and vitamin $D_2$) or 10 μM (1α(OH)$D_3$, and 1α(OH)$D_2$), and then incubated at 37 °C with shaking at 200 or 1800 rpm for 24 h. After the reaction, the cell suspension was extracted with four volumes of chloroform/methanol (3:1 v/v). The organic phase was recovered and dried up. The resulting residue was solubilized with acetonitrile and subjected to HPLC as described in the section of *HPLC analysis of metabolites.*

### 2.4. UV irradiation of the recombinant yeast cells

The recombinant *S. cerevisiae* cells suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 4% glucose and 0.2% 3-hydroxy-β-cyclodextrin were irradiated in the beaker using a UV-B lamp (Sankyo Denki, G15T8E, 15W, Japan) at a distance of 22 cm at 25 °C for 0, 0.25, 0.5, 1.0, 1.5, 2, 4, 6 or 8 h.

### 2.5. Production of 25(OH)$D_2$ by UV-irradiated recombinant S. cerevisiae cells expressing human CYP2R1 without substrate addition

After UV irradiation, the cells suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 4% glucose were incubated at 37 °C for 24 h, and extracted with four volumes of chloroform/methanol (3:1 v/v). Samples were then analyzed by HPLC as detailed below.

### 2.6. HPLC analysis of metabolites

HPLC was performed on a YMC-Pack ODS-AM [4.6 × 300 mm] (YMC Co., Tokyo, Japan). A linear gradient of 70–100% acetonitrile aqueous solution for 15 min followed by 100% acetonitrile for 25 min was used as a mobile phase. The flow-rate was 1.0 mL/min, and the elution was detected by measuring absorption at 265 nm. The column temperature was maintained at 40 °C.

### 2.7. Mass spectrometric analysis of the metabolites

Isolated metabolites from HPLC effluents were subjected to LC–mass spectrometric analysis using a Finnegan LCQ-70 Advantage Mix (Thermo Fisher Scientific, Waltham, MA) in atmospheric pressure chemical ionization (APCI), positive mode. The conditions of LC were as follows: column, reverse phase ODS column (2 × 150 mm) (Develosil ODS-HG-3; Nomura Chemical Co., Ltd., Aichi, Japan); mobile phase, linear gradient of 20–100% acetonitrile aqueous solution for 25 min followed by 100% acetonitrile for 12 min; flow rate, 0.2 mL/min; UV detection; 265 nm.

### 2.8. Other methods

The concentrations of vitamin $D_3$ derivatives were estimated by their molar extinction coefficient of 1.8 × $10^4$ $M^{-1}cm^{-1}$ at 264 nm [9].

## 3. Results

### 3.1. Human CYP2R1-dependent metabolism of vitamin $D_3$, $D_2$, 1α(OH)$D_3$, and 1α(OH)$D_2$ in the recombinant S. cerevisiae cells

As described previously, human CYP2R1 was successfully expressed in *S. cerevisiae* cells [4]. Fig. 2A shows the HPLC profiles of the substrates vitamins $D_3$ and $D_2$, and their metabolites in the reaction buffer containing 0.2% 3-hydroxy-β-cyclodextrin. The major metabolites had the same retention times as 25(OH)$D_3$ and 25(OH)$D_2$, respectively. These metabolites were not observed in the control AH22/pGYR cells, suggesting that the metabolism vitamins $D_3$ and $D_2$ is dependent on CYP2R1. The metabolites collected from the HPLC effluents were subjected to LC–mass spectrometric analysis. The mass spectrum of the metabolite of vitamin $D_3$ showed a molecular ion at *m/z* 401 (M+H, 44%), and fragment ions at 383 (M+H-$H_2O$, 100%) and 365 (M+H-2$H_2O$, 24%). This spectrum coincided with the spectrum of the authentic standard of 25(OH)$D_3$. On the other hand, the mass spectrum of the metabolite of vitamin $D_2$ showed a molecular ion at *m/z* 413 (M+H, 100%), and a fragment ion at 395 (M+H-$H_2O$, 100%). This mass spectrum coincided with that of the authentic standard of 25(OH)$D_2$. These results strongly suggest that the major metabolites of vitamin $D_3$ and $D_2$ were 25(OH)$D_3$ and 25(OH)$D_2$, respectively. Fig. 2B shows the HPLC profiles of 1α(OH)$D_3$, and 1α(OH)$D_2$ and their metabolites. The retention times of these metabolites are identical with those of 1α,25(OH)$_2D_3$, and 1α,25(OH)$_2D_2$, respectively, and LC-mass analysis confirmed that the metabolites

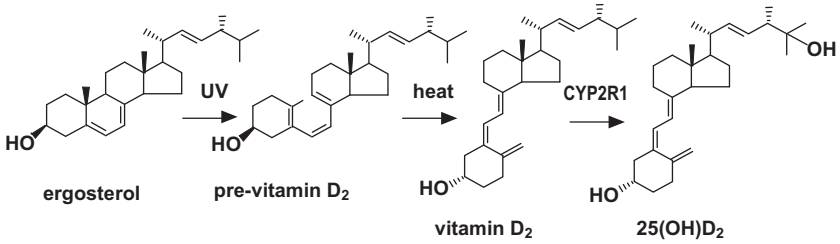


**Fig. 1.** Pathway to produce 25(OH)$D_2$ from endogenous ergosterol in the recombinant *S. cerevisiae* cells expressing human CYP2R1.

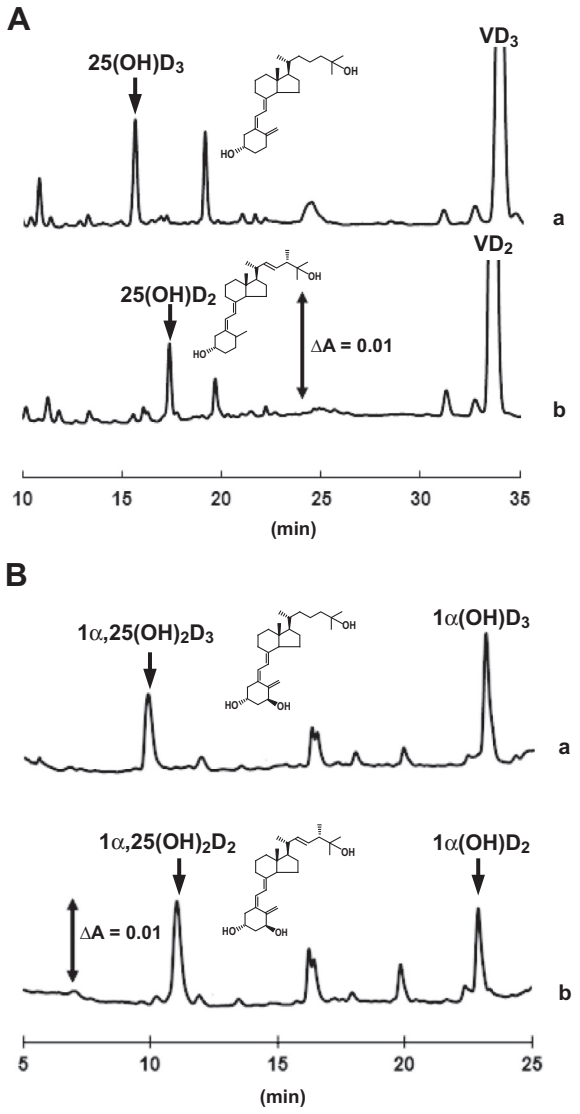


**Fig. 2.** HPLC profiles of the substrates vitamin $D_3$ (A-a) and vitamin $D_2$ (A-b) or 1α(OH)$D_3$ (B-a) and 1α(OH)$D_2$ (B-b) and their metabolites in the CYP2R1-expressing *S. cerevisiae* cells. The arrows indicate CYP2R1-dependent metabolites.

are 1α,25(OH)$_2D_3$, and 1α,25(OH)$_2D_2$. The conversion ratios of 10 μM vitamin $D_3$ and vitamin $D_2$ to their 25-hydroxylated products were 9.6% and 4.5%, respectively; thus, the final concentrations of 25(OH)$D_3$ and 25(OH)$D_2$ were calculated to be 0.96 and 0.45 μM, respectively. The conversion ratios of 50 μM of vitamin $D_3$ or vitamin $D_2$ to their 25-hydroxylated products were 4.3% and 2.4%, respectively. Thus, the final concentrations of 25(OH)$D_3$ and 25(OH)$D_2$ are calculated to be 2.2 and 1.2 μM, respectively. In contrast, conversion ratios of 10 μM of 1α(OH)$D_3$ and 1α(OH)$D_2$ to their 25-hydroxylated products were 49% and 57%, respectively. Thus, the final concentration of 1α,25(OH)$_2D_3$ and 1α,25(OH)$_2D_2$ were calculated to be 4.9 μM, 5.7 μM, respectively. Note that these values were much higher than the 25(OH)$D_3$ and 25(OH)$D_2$ from 10 μM of vitamin $D_3$ and vitamin $D_2$.

### 3.2. Production of 25(OH)$D_2$ by UV-irradiated recombinant S. cerevisiae cells expressing human CYP2R1 without additional substrate

Fig. 3 shows vitamin $D_2$ and 25$D_2$ formation in the recombinant *S. cerevisiae* cells with a 2 h-UV irradiation and successive 24 h-incubation at 37 °C. Instead, the amount of ergosterol was

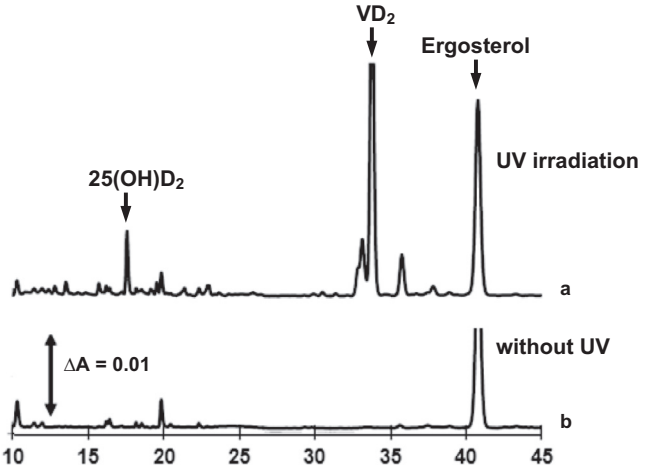


**Fig. 3.** HPLC profiles of ergosterol, vitamin $D_2$, and 25(OH)$D_2$ in the recombinant *S. cerevisiae* cells expressing human CYP2R1 cells. After 2 h-UV irradiation and successive 24 h-incubation at 37 °C, the extract in organic solvent was analyzed by HPLC as described in Section 2.

significantly reduced when compared to the recombinant *S. cerevisiae* cells that were not UV irradiated, suggesting that the endogenous ergosterol was converted to vitamin $D_2$ by UV irradiation and thermal isomerization. The final concentration of 25(OH)$D_2$ was estimated to be 3.3 μM. Note that this value is higher than the 1.2 μM that was converted from the vitamin $D_2$ when 50 μM was added as a substrate.

### 3.3. Relationship between vitamin $D_2$ or 25(OH)$D_2$ formation and UV irradiation time

The amount of vitamin $D_2$ in *S. cerevisiae* cells linearly increased with increasing UV irradiation time periods as shown in Fig. 4A. However, the amount of 25(OH)$D_2$ was increased up to 2 h, but then decreased (Fig. 4B). These results suggest that a long-term UV irradiation inhibits the CYP2R1-dependent 25-hydroxylation activity.

## 4. Discussion

As mentioned above, the periodic measurement of serum 25(OH)D concentration appears to be important in maintaining good health by preventing osteoporosis and some cancers. The serum 25(OH)$D_3$ level is generally much higher than the level of 25(OH)$D_2$. However, serum 25(OH)$D_2$ concentration is sometimes not neglected because edible mushroom species contain significant amounts of vitamin $D_2$. Sun-dried mushrooms in particular contain high levels of vitamin $D_2$. Thus, the serum 25(OH)$D_2$ concentration in people who usually ingest foods and/or supplements containing vitamin $D_2$ must be measured. Note that mushroom vitamin $D_2$ is produced from ergosterol by UV irradiation, as with the vitamin $D_2$ that is produced in the *S. cerevisiae* cells in this study. The quantification of serum 25(OH)$D_3$ and 25(OH)$D_2$ concentrations by LC–MS analysis requires authentic 25(OH)$D_3$ and 25(OH)$D_2$ standards. Thus, if the periodic measurement of serum 25(OH)$D_3$ and 25(OH)$_2$ concentrations were to be required for all workers and elderly people to prevent osteoporosis, some cancers, and chronic diseases throughout the world, then the market for 25(OH)$D_3$ and 25(OH)$D_2$ would dramatically increase.

Our previous reports demonstrated that the R73V/R84A variant of *Streptomyces gliseolus* CYP105A1 was able to sequentially catalyze 25-, 1α, and 26-hydroxylations [10,11]. Yasutake et al. [12]

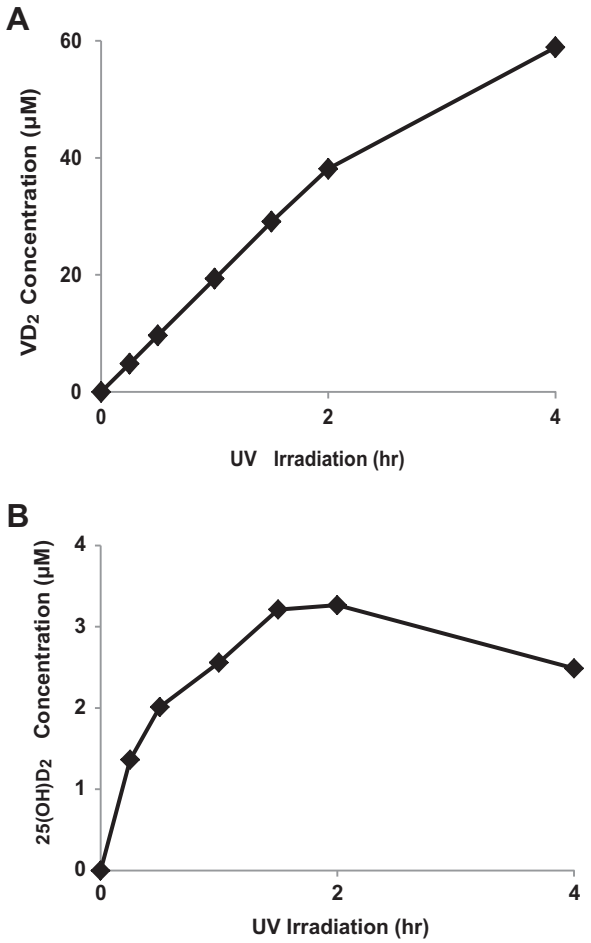


**Fig. 4.** Time-course of the vitamin $D_2$ concentrations in the whole cell culture of the yeast cells expressing human CYP2R1 (A), and production of 25(OH)$D_2$ for 24 h after the designated UV irradiation time (B).

also observed a two-step hydroxylation of vitamin $D_3$ by CYP107. Mammalian CYP27A1 also catalyzed a multi-step hydroxylation of vitamin $D_3$ and vitamin $D_2$ [5]. In contrast, human CYP 2R1 only caused a 25-hydroxylation towards vitamin $D_3$ and vitamin $D_2$. Thus, the enzymatic properties of CYP2R1 are quite appropriate for the production of 25(OH)$D_3$ and 25(OH)$D_2$.

For our first step, we added vitamin $D_3$ or vitamin $D_2$ that was dissolved in ethanol to the cell suspensions and they were incubated with shaking at 200 rpm, but their 25-hydroxylated vitamin products were barely produced most likely because of poor cell uptake. In our previous studies, the remarkable effect of 2-hydroxypropyl-β-cyclodextrin (HPCD) was observed in the conversion of vitamin $D_3$ to its hydroxylated products using recombinant *Streptomyces lividans* cells expressing R73V/R84A variant of CYP105A1 [11]. We also examined the effect of HPCD using recombinant yeast expressing CYP2R1. As expected, the addition of HPCD markedly enhanced CYP2R1-dependent vitamin D 25-hydroxylation. Bulky and hydrophobic vitamin D could be lodged inside cyclodextrin ring thereby enhancing its solubility in aqueous solution. The reaction was also performed with shaking at 1800 rpm in the absence of HPCD. Under this condition, productivity of the 25-hydroxylated vitamins was nearly the same as that in the presence of HPCD. Thus, addition of HPCD or shaking at high speeds appeared to help microorganisms take up vitamin D. Because vigorous shaking is difficult in a large-scale production, the use of HPCD is needed in a large-scale production. It should be noted that 1α(OH)$D_3$ and 1α(OH)$D_2$ were more efficiently converted to their 25-hydroxylated products than vitamin $D_3$ and vitamin $D_2$ as shown in Fig. 2. However, our previous studies using microsomal fractions prepared from CYP2R1-expressing yeast cells demonstrated that vitamin $D_3$ ($D_2$) and 1α(OH)$D_3$ (1α(OH)$D_2$) were similarly good substrates for CYP2R1 [4]. Therefore, the remarked difference between vitamin $D_3$ (or $D_2$) and 1α(OH)$D_3$ (or 1α(OH)$D_2$) in the conversion ratios is not based on enzymatic properties of CYP2R1 but based on their intake efficiency.

Yeast cells are known to produce ergosterol, which could be converted to pre-vitamin $D_2$ by UV irradiation and further converted to vitamin $D_2$ by thermal isomerization. Thus, we irradiated the recombinant yeast cells expressing human CYP2R1 with UV to form previtamin $D_2$. An incubation at 37 °C induced isomerization of previtamin $D_2$ to vitamin $D_2$, which was then converted it to 25(OH)$D_2$ by CYP2R1. One remarkable feature of this method is that there is no need for substrate addition. Judging from the vitamin $D_2$ and 25(OH)$D_2$ concentrations, the vitamin $D_2$ that was generated in yeast cells was efficiently converted to 25(OH)$D_2$ by CYP2R1 (Fig. 4). For example, after 2 h of UV irradiation, 24 h of incubation produced 3.3 μM of 25(OH)$D_2$ and the vitamin $D_2$ concentration was 38 μM. Thus, conversion ratio from vitamin $D_2$ to 25(OH)$D_2$ was calculated to be 8.7%. This value is significantly higher than 2.4% conversion ratio that resulted from the addition of 50 μM of substrate vitamin $D_2$. As shown in Fig. 4, the production of 25(OH)$D_3$ had a maximum at 2 h-UV irradiation, and then gradually decreased with increasing UV irradiation time. Currently, the reason for this trend is unclear, but CYP2R1-dependent activity requires NADPH, NADPH-P450 reductase, and CYP2R1. Ultraviolet irradiation might induce oxidative damages to NADPH-P450 reductase, and/or CYP2R1. In mammals and yeast, UVB radiation induces NADPH oxidase activity and reactive oxygen species (ROS) generation [13,14]. Thus, the NADPH level in the cell may be decreased by UVB radiation, which results in activity, and ROS might inactivate NADPH-P450 reductase, and/or CYP2R1.

In conclusion, we have established a novel method for producing 25(OH)$D_2$ by using recombinant *S. cerevisiae* cells expressing human CYP2R1. Approximately, 1.3 mg of 25(OH)$D_2$ was produced from 1 g of yeast cells (dry weight). The reduction of UV-dependent ROS formation and/or enzyme inactivation would increase 25(OH)$D_2$ production.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.124.

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