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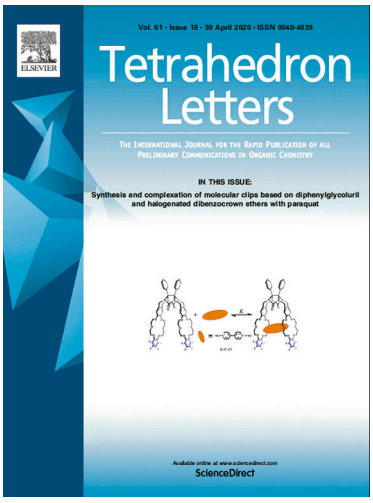

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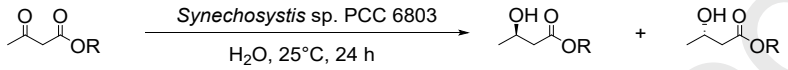

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Asymmetric visible-light photobiocatalytic reduction of β-keto esters utilizing the cofactor recycling system in *Synechocystis* sp. PCC 6803

Shusei Tanaka[a] , Hideo Kojima[a,] * , Satomi Takeda[b] , Rio Yamanaka[c] , and Tetsuo Takemura[d]

[a]*Department of Chemistry, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*
[b]*Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan*
[c]*Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, 7-2-1 Kamiono, Himeji, Hyogo 670-8524, Japan*
[d]*Faculty of Science, Josai University, 1-1 Keyakidai, Sakadoi, Saitama 350-0295, Japan*

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* Corresponding author. Department of Chemistry, Graduate School of Science, Osaka Prefecture University; e-mail: kojima@c.s.osakafu-u.ac.jp

ABSTRACT

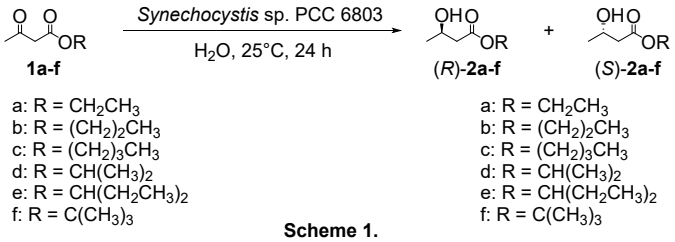
The asymmetric reduction of β-keto esters employing a wild-type strain of cyanobacterium *Synechocystis* sp. PCC 6803 under illumination of red LED light at 25 °C for 24 h was evaluated. As a result, the corresponding (*R*)-β-hydroxy esters were obtained as major products. The *R*-selectivity was shown to increase for bulkier substrates. Moreover, it was also found that the *R*-selectivity increased with decreasing substrate concentrations. This can be explained by the assumption that the K_m value of the *R*-selective reductase is smaller than that of the *S*-selective enzyme involved in the reaction. Additionally, it was demonstrated that the *R*-selective reductase required the light-dependent production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for effective reaction; however, the *S*-selective variant did not. Overall, cyanobacterium was employed as a sustainable photobiocatalyst proliferating under illumination of light, while utilizing inorganic salts and atmospheric carbon dioxide (CO_2). Employing the whole-cell system allowed for the preparation of industrially-important chiral compounds, such as optically active β-hydroxy esters.

Due to excellent enantioselectivities of enzymes inside the cells, whole-cell asymmetric reduction of ketones is a particularly powerful tool for the production of chiral alcohols.[1] Although reductive biocatalysis is a promising method for optically active alcohols, the method has a fundamental problem; in this method, the redox coenzyme is used for reduction and is oxidized according to the reduction of a ketone. Therefore, it is necessary to regenerate the oxidized coenzyme to the reduced form. Recently, asymmetric visible-light photocatalytic methods have attracted increased attention due to the necessity to develop eco-friendly techniques for biotransformation.[2] In the photobiocatalytic method, the redox cofactor nicotinamide adenine dinucleotide phosphate (NADPH) acting as a reducing agent is regenerated by a photosynthetic system under illumination of visible-light.[3] We have previously reported that whole-cells of plants, such as *Nicotiana tabacum*[4] and *Arabidopsis thaliana*,[5] worked as effective photobiocatalysts for the preparation of β-hydroxy esters, versatile chiral building blocks in the pharmaceutical, agrochemical, and food industries,[6] by the asymmetric reduction of β-keto esters.

Similarly to plants, cyanobacteria are photosynthetic organisms; however, their potential as whole-cell photobiocatalysts for the production of various chemicals is considerably higher than that of plants.[7] Cyanobacteria are excellent sustainable catalysts, as they proliferate more efficiently under illumination of solar light than plants, while utilizing inorganic salts and atmospheric carbon dioxide (CO_2).[7a] Thus, using techniques employing cyanobacteria as photobiocatalysts is expected to contribute to energy-saving and CO_2 reduction. A number of studies considering the reduction of C=C bonds[8] and C=O bonds[9] utilizing cyanobacteria have been reported. Nonetheless, only a few reports on the synthesis of β-hydroxy esters using cyanobacteria as photobiocatalysts have been described. Takemura *et al.* investigated the synthesis of *tert*-butyl (*S*)-3-hydroxybutanoate from *tert*-butyl 3-oxobutanoate utilizing knockout mutants of cyanobacterium *Synechocystis* sp. PCC 6803, which is a genus of fresh water cyanobacteria.[10] In the present study, we evaluated the factors controlling the stereoselectivity of the asymmetric reduction of β-keto esters using a wild-type strain of *Synechocystis* sp. PCC 6803 as a photobiocatalyst.

Ito *et al.* reported that employing a red light-emitting diode (LED) lamp as the light source resulted in a more effective asymmetric reduction of 2',3',4',5',6'-pentafluoroacetophenone using *Synechocystis* sp. PCC 6803 than utilizing blue or green LED light.[9b] Consequently, we employed a red LED lamp as a light source during the reactions. In the first instance, we investigated the effect of steric hindrance of the substrate on the reaction. The reactions of β-keto esters **1a-f** (10 μg/mL) containing ester moieties of various bulkiness, were carried out in the presence of *Synechocystis* sp. PCC 6803 under illumination of red LED light (660 nm, 10 μmol photons m^{-2} s^{-1}) at 25°C for

values were determined by gas chromatography (GC). All values are the mean of three experimental results (Table 1).



Scheme 1.

In all cases, the corresponding (*R*)-*β*-hydroxy esters **2a-f** were obtained in moderate yields, as shown in Table 1. Notably, in the case of *β*-hydroxy esters **2a-c** possessing straight-chain ester moieties, the yields of (*R*)-**2** increased considerably with the increase of the number of carbon atoms in the chains (Table 1, entries 1-3). On the other hand, for the *β*-hydroxy esters **2d-f** containing branched-chain ester functionalities, the highest ee value was achieved in the case of **1f**, probably due to the large steric hindrance of the bulky *tert*-butyl group (Table 1, entries 4-6). Hence, overall, the *R*-selectivity tended to increase for bulkier substrates.

We subsequently changed the substrate concentration of **1f** from 10 μg/mL to 50 μg/mL and 250 μg/mL, which resulted in a decrease in the ee values from 75% to 57% and 39%, respectively. Thus, the *R*-selectivity was higher at lower substrate concentrations. Takemura *et al.* have previously reported that 3-oxyacyl-[acyl-carrier-protein]reductase (the Slr0315 protein) was the major *R*-selective reductase in the asymmetric reduction of *tert*-butyl 3-oxobutanoate using *Synechocystis* sp. PCC 6803.[10] Moreover, it was determined that the stereoselectivity could be regulated by changing the substrate concentration in the microbial reduction of ketones due to the difference in the Michaelis constants (K_m) of the *R*-selective enzyme and the *S*-selective enzyme in the Michaelis-Menten kinetic model. At low substrate concentrations, the enzyme with the smallest K_m value catalyzed the reaction preferentially over other enzymes with larger K_m values.[11] Based on these findings, it is considered that the K_m value of the *R*-selective 3-oxyacyl-[acyl-carrier-protein]reductase was also smaller than that of the *S*-selective reductase involved in the reaction.

Table 1. Asymmetric reduction of *β*-keto esters **1a-f** using a wild-type strain of cyanobacterium *Synechocystis* sp. PCC 6803.[a]

Entry	Substrate	Product, %[b]	(*R*)-**2**, %	(*S*)-**2**, %	ee % (*R*)[b]
1	**1a**	**2a**, 16	10	6	27
2	**1b**	**2b**, 76	63	13	66
3	**1c**	**2c**, 96	79	17	64
4	**1d**	**2d**, 53	36	17	34
5	**1e**	**2e**, 95	82	13	73
6	**1f**	**2f**, 89	84	5	75

[a] Reaction conditions: **1** (10 μg/mL); cyanobacteria ($Abs_{680-750}$ 0.33); red LED light (660 nm, 10 μmol photons m^{-2} s^{-1}); 25°C, 24 h.

[b] Determined by GC. All values are the mean of three experimental results (n = 3).

Subsequently, we investigated the effects of light on the progress of the reaction. The reaction of **1f** was carried out under illumination of light with intensity of 0.5-20 μmol photons m^{-2} s^{-1}. The yields of (*R*)-**2f** and (*S*)-**2f** obtained in each reaction are increased with the increase in the light intensity (photon flux density: PFD) up to 2.5 μmol photons m^{-2} s^{-1} and stayed high thereafter. On the other hand, the yield of (*S*)-**2f** decreased a little up to 2.5 μmol photons m^{-2} s^{-1} and stayed low thereafter. These results implied that the *R*-selective reductase required the light-dependent production of NADPH, whereas the *S*-selective reductase did not. It was previously reported that the reduction of exogenous ketones, such as acetophenone derivatives, using cyanobacterium *Synechococcus* sp. PCC 7942, depended on reduced NADPH. The cofactor typically acts as a reducing agent during anabolic reactions in the cells and is recycled during the photosynthesis in photosynthetic organisms.[9d] Similarly, the asymmetric reduction of *β*-ketoesters **1** by *Synechocystis* sp. PCC 6803 may be controlled by the amount of NADPH in the cells. At 2.5 μmol photons m^{-2} s^{-1} or higher, the yield of (*R*)-**2f** remained nearly constant, possibly because a sufficient quantity of NADPH was produced by the photosynthetic system at those light intensities. Thus, the described reaction is eco-friendly, as the use of low intensity and low energy red-light was sufficient to achieve effective transformations.

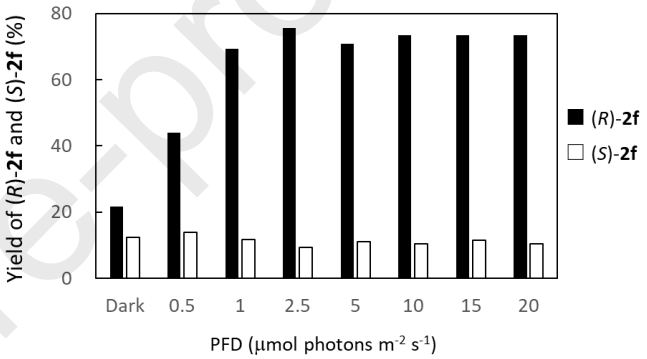


Figure 1. The effect of light during the reaction of **1f**. Reaction conditions: **1f** (10 μg/mL); cyanobacteria ($Abs_{680-750}$ 0.33); 25 °C, 24 h. All values are the mean of three experimental results (n = 3).

It was previously described that the ratio of NADPH to reduced nicotinamide adenine dinucleotide (NADH) under light conditions was 1.7-fold higher than that under dark conditions in cyanobacterial cells.[12] To further investigate the effect of the quantity of NADPH on the yield of (*R*)-**2f**, we examined the influence of light during the precultivation stage, prior to the reaction. Thus, the precultivation was carried out for 2 days in the dark or under illumination of fluorescence light (20 μmol photons m^{-2} s^{-1}) before conducting the reaction. The asymmetric reduction of **1f** was subsequently performed in the dark or under illumination of red LED light (660 nm, 10 μmol photons m^{-2} s^{-1}) at 25 °C for 3 h. For the dark reactions, the yield of (*R*)-**2f** in the light precultivation were slightly higher than those in the dark precultivation (DD *vs.* LD in Figure 2). On the other hand, for the light reactions, the light conditions at the precultivation stage did not have a significant effect on the yield of (*R*)-**2f** (DL *vs.* LL in Figure 2). Thus, the yield of (*R*)-**2f** increased greatly under illumination of light during the reaction (DD *vs*. DL and LD *vs*. LL in Figure 2). These results suggest that (*R*)-**2f** was produced by NADPH, which was regenerated under illumination of light during the precultivation stage or during the reaction.

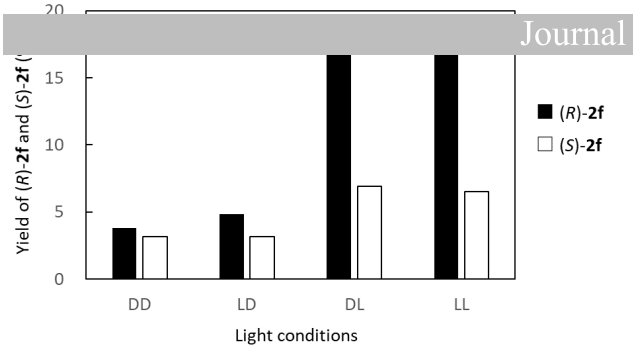


Figure 2. The effect of light at the precultivation stage or during the reaction. Precultivation conditions: 25 °C; 2 days; fluorescence light (20 μmol photons m^{-2} s^{-1}) or dark. Reaction conditions: **1f** (10 μg/mL); cyanobacteria ($Abs_{680-750}$ 0.33); 25 °C, 3 h; red LED light (660 nm, 10 μmol photons m^{-2} s^{-1}) or dark. DD, dark in the precultivation stage-dark during the reaction; LD, light-dark; DL, dark-light; LL, light-light. All values are the mean of three experiments (n = 3).

Furthermore, we also investigated the effect of the quantity of cyanobacterial cells on the progress of the reactions (Table 2). The quantity of cells was adjusted on the basis of the absorbance at 680 nm in the ultraviolet (UV)-visible absorption spectrum of cyanobacteria dispersed in aqueous solution, setting the absorbance at 750 nm to zero ($Abs_{680-750}$). The yields and ee values of **2f** increased with the increasing quantity of cells for $Abs_{680-750}$ of 0.99 or bellow (Table 2, entries 1-5). Moreover, for $Abs_{680-750}$ of 0.99, the yield and ee values increased to 96% and 89%, respectively (Table 2, entry 5). In the presence of a low quantity of cells, the yield and ee values were also low, possibly because of cell damage caused by the excess amounts of substrates (Table 2, entries 1 and 2).

Furthermore, for $Abs_{680-750}$ equal to 2.97, the ee value decreased to 57% (Table 2, entry 7). This may be a consequence of unequal exposure of the cells to light. The phenomenon of self-shadowing has previously been reported for high-cell-density cultures. It results in a lower amount of light reaching the microorganisms, and thus a lower metabolic rate.[13] Moreover, in the present study, it was found that the use of LED light of 60 μmol photons m^{-2} s^{-1} in the case of $Abs_{680-750}$ of 1.32 led to the increase in the ee value from 67% to 77% (Table 2, entries 6 and 8). This outcome also suggested that NADPH generated by light was an essential cofactor for the production of (*R*)-**2f**.

Table 2. Effect of the quantity of cyanobacterial cells.[a]

Entry	Cyanobacteria, $Abs_{680-750}$	**2f**, %[b]	(*R*)-**2f**, %	(*S*)-**2f**, %	ee, % (*R*)[b]
1	0.033	34	24	10	40
2	0.066	44	33	11	50
3	0.33	89	78	11	75
4	0.66	93	85	8	82
5	0.99	96	91	5	89
6	1.32	99	83	16	67
7	2.97	100	79	21	58
8[c]	1.32	98	87	11	77

[a] Reaction conditions: **1f** (10 μg/mL); 25°C, 24 h; red LED light (660 nm, 10 μmol photons m^{-2} s^{-1}).

[b] Determined by GC. All values are the mean of three experiments (n = 3).

[c] Red LED light (660 nm, 60 μmol photons m^{-2} s^{-1}).

he asymmetric reduction of *β*-keto esters **1** using a wild-type strain of cyanobacterium *Synechocystis* sp. PCC 6803 under illumination of red LED light (660 nm) at 25°C for 24 h. As a result, the corresponding (*R*)-*β*-hydroxy esters **2** were successfully obtained. We determined that the *R*-selectivity tended to increase for bulkier substrates. Moreover, the *R*-selectivity increased with decreasing substrate concentrations. This can be explained by assuming that the K_m value of the *R*-selective reductase is smaller than that of the *S*-selective enzyme involved in the reaction. Moreover, we have demonstrated that the *R*-selective reductase required the light-dependent production of NADPH, while the *S*-selective reductase did not. Notably, the described photobiocatalytic system is sustainable, as the cyanobacteria proliferate under illumination of light, while utilizing inorganic salts and atmospheric CO_2. Thus, the use of cyanobacteria as whole-cell photobiocatalysts allowed for efficient preparation of industrially-relevant chiral compounds, such as optically active *β*-hydroxy esters.

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Supplementary Material

Supplementary data to this article can be found online at https://.

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Highlights

- Asymmetric reduction of *β*-keto esters using a cyanobacterium
- (*R*)-*β*-Hydroxy esters obtained as major products
- *R*-selective reductase with the light-dependent production of NADPH
- Sustainable photobiocatalyst utilizing the cofactor recycling system