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BIOCATALYSIS

Production of chiral alcohols from prochiral ketones by microalgal photo-biocatalytic asymmetric reduction reaction

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Abstract Microalgal photo-biocatalysis is a green technique for asymmetric synthesis. Asymmetric reduction of nonnatural prochiral ketones to produce chiral alcohols by microalgal photo-biocatalysis was studied in this work. Acetophenone (ACP) and ethyl acetoacetate (EAA) were chosen as model substrates for aromatic ketones and β-ketoesters, respectively. Two prokaryotic cyanophyta and two eukaryotic chlorophyta were selected as photo-biocatalysts. The results proved that nonnatural prochiral ketones can be reduced by microalgal photo-biocatalysis with high enantioselectivity. Illumination is indispensable to the photo-biocatalysis. For aromatic ketone, cyanophyta are eligible biocatalysts. For ACP asymmetric reduction reaction, about 45% yield and 97% *e.e.* can be achieved by the photo-biocatalysis reaction with *Spirulina platensis* as biocatalyst. On the contrary, chlorophyta are efficient biocatalysts for β-ketoester asymmetric reduction reaction among the four tested algae. For EAA asymmetric reduction reaction, about 70% yield and 90% *e.e.* can be achieved with *Scenedesmus obliquus* as biocatalyst. The microalgae used in this study outperformed other characterized biocatalysts such as microbial and plant cells.

Keywords Asymmetric reduction · Photo-biocatalysis · Chiral alcohol · Microalgae

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Introduction

Because of safety, therapeutic, and regulatory concerns, there has been increasing interest in the development of processes capable of producing enantiomerically pure pharmaceuticals, agrochemicals, and other fine chemicals [9, 23]. Enantiomerically pure chiral products are usually synthesized from chiral building blocks, such as chiral alcohols and chiral amines. Chiral alcohols are the most important chiral building blocks due to their unique structural properties [5, 22, 23, 27]. Asymmetric reduction of the corresponding prochiral ketones is an efficient and promising route to produce chiral alcohols [12]. The asymmetric reduction reaction by biocatalysis, especially by whole-cell biocatalysis, has many advantages such as outstanding enantioselectivity, mild reaction conditions and environmental friendliness, and regeneration of cofactor [i.e., nicotinamide adenine dinucleotide phosphate, NAD(P)H] in situ in whole cells [16, 22, 25, 28]. So far, bacteria, fungi, and plant tissues have been extensively researched as biocatalysts [3, 6, 11, 16, 22, 29, 34], and many excellent bioreaction processes have been developed. In our previous work, we devoted our efforts to explore the asymmetric reduction of aromatic ketone and β-ketoesters by yeast and plant tissues [31, 34], and we also developed bioreduction processes by introducing resin adsorption and ionic liquid or organic two-phase systems [30, 32, 33, 35]. However, for these biocatalysts, reduced state cosubstrates, such as glucose or alcohol, are required for cofactor regeneration. If such addition of reduced state cosubstrate can be eliminated, asymmetric reductions will be much more environmentally friendly and economical.

Photosynthesis provides a possible route to resolve this problem. Utilizing light energy, NAD(P)H can be regenerated through photosynthesis. Especially, single-cell

microalgae are good photo-biocatalyst candidates for the asymmetric reduction reaction due to the following two merits: Firstly, they are easy to manipulate and have high growth rate. Secondly, they have good ecological benefits, as microalgae have been used for feed and renewable bioenergy production [2, 26], and can also be used to mitigate CO_2 emissions [13]. We think that microalgae are efficient biocatalysts for asymmetric reduction reactions, since there are plentiful intracellular oxidoreductases in microalgal cells. Previously, cyanobacteria, *Synechococcus* sp. PCC 7942, was initially used as biocatalyst to reduce aryl methyl ketones [17], showing excellent enantioselectivity. Subsequently, there have been a few reports regarding microalgal photo-biocatalytic asymmetric reduction reactions [7, 10], but most of these reports focused on cyanobacteria, mainly *Synechococcus* sp. PCC 7942. However, information on photo-biocatalytic asymmetric reduction reaction by other microalgae, such as eukaryotic chlorophyta, is scarce.

Developing novel high-efficiency biocatalysts and processes is the first important and essential step towards biological asymmetric synthesis. The objective of this study is to explore the possibility of asymmetric reduction of aromatic ketones and β-ketoesters by microalgal photo-biocatalytic reaction. Acetophenone (ACP) and ethyl acetoacetate (EAA) are chosen as model substrates for aromatic ketones and β-ketoesters, respectively, which are widely applied in practical asymmetric synthesis. Four microalgae, *Spirulina platensis*, *Anabaena flosaquae*, *Scenedesmus obliquus*, and *Chlorella vulgaris*, were chosen as photo-biocatalysts. The first two are prokaryotic cyanophyta, and the others are eukaryotic chlorophyta.

Materials and methods

Chemicals

ACP and benzaldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd. (China) as analytical-grade reagents. *R*- and *S*-1-phenylethanol (PEA), EAA, ethyl (*S*)-3-hydroxybutyrate (*S*-EHB), and ethyl (*R*)-3-hydroxybutyrate (*R*-EHB) were purchased from ACROS Organic In. (New Jersey, USA) as laboratory-grade reagents. Ethyl acetate and other reagents were of analytical grade and commercially available.

Microalgae and culture medium

S. platensis, *A. flosaquae*, *S. obliquus*, and *C. vulgaris* were used in this work. They were purchased from Institute of Hydrobiology (IHB), Chinese Academy of Sciences (CAS).

For culture of these microalgae, the following culture media were used: Spirulina medium was used for *S. platensis* culture [1], BG-11 medium was used for *A. flosaquae* culture [21], and modified soil extract (SE) medium was used for *S. obliquus* and *C. vulgaris* culture [13].

General procedure for asymmetric reduction of ketones with microalgae

Five milliliters of the four kinds of microalgal seed culture broth (OD_{685} about 3.0, where OD_{685} is the optical density at 685 nm, used to indicate the microalgal biomass density based on turbidimetry) was inoculated into a 200-mL bubble column photobioreactor containing 100 mL of the corresponding culture medium. They were incubated at 28°C with 9,000 lux illumination on the photobioreactor surface provided by a continuous cool white fluorescent light. A 5% CO_2 gas (v/v, mixed with air), provided by a gas cylinder, was aerated from the photobioreactor bottom at rate of 0.1 v/v min^{-1} (volume gas per volume broth per minute). When the cell density reached $OD_{685} = 3.0$, a certain amount of substrate (ACP or EAA) was added to the culture broth to a set concentration. For ACP, the final concentration was 5 mmol/L, and for EAA, it was 20 mmol/L. The reaction was carried out under 28°C and 9,000 lux illumination for a certain period (48 h for ACP reduction reaction, 72 h for EAA reduction reaction) to obtain a favorable conversion degree. Finally, the reaction mixture was extracted with ethyl acetate. The organic phase was dried with anhydrous Na_2SO_4. Then, the concentrations of product and substrate were determined, and the chemical yield and enantioselectivity were evaluated. Each experiment was replicated in parallel at least three times, and the average and standard deviation were calculated.

Analysis

The concentrations of ACP, *R*- and *S*-PEA were determined by gas chromatography (GC, model 6890; Agilent Technologies Co., Ltd.) equipped with a chiral Cyclodex-B capillary column, using benzaldehyde as internal standard. The GC conditions were N_2 carrier gas with 3.5 mL/min flow rate, splitting ratio of 50:1, flame ionization detector (FID), and the following oven temperature program: 90°C for 5 min, increasing from 90°C to 180°C at rate of 15°C/min, and holding at 180°C for 2 min [34].

The analytical approach for EAA reduction reaction was modified from the approach using ethyl 4-chloro-3-oxobutanoate and chiral ethyl 4-chloro-3-hydroxybutanoate in our previous work [30]. The concentrations of EAA and EHB were determined by GC equipped with an HP-5 ms (0.25 mm × 30 m) capillary column and a FID. Decane

was used as internal standard. The GC conditions were N_2 carrier gas with flow rate of 3 mL/min, splitting ratio of 50:1, and the following oven temperature program: 90°C for 4 min, increasing from 90°C to 160°C at rate of 10°C/min, and holding at 160°C for 2 min. *S*-EHB and *R*-EHB were determined by high-performance liquid chromatography (HPLC, model 1100; Agilent Technologies Co., Ltd.) equipped with a Chiralcel OB column (4.6 mm × 250 mm) (Daicel Chemicals, Japan). The HPLC conditions were hexane/isopropyl alcohol (9/1, v/v) as mobile phase, flow rate of 1.5 mL/min, ambient column temperature, and ultraviolet (UV) detection set at 215 nm.

The reaction degree and enantioselectivity are indicated by yield (chemical yield, %) and *e.e.* (enantiomeric excess, %), respectively, defined as

$$\text{yield} = \frac{C_P}{C_O} \times 100, \quad (1)$$

$$e.e. = \frac{C_S - C_R}{C_S + C_R} \times 100, \quad (2)$$

where C_0 is the initial substrate concentration, C_P is the final product concentration, C_S is the final *S*-form product concentration, and C_R is the final *R*-form product concentration.

Results and discussion

Asymmetric reduction of ACP and EAA catalyzed by prokaryotic cyanophyta and eukaryotic chlorophyta was investigated. Four microalgae, *S. platensis*, *A. flosaquae*, *S. obliquus*, and *C. vulgaris*, were chosen as biocatalysts. The conjectured reaction scheme of the photo-biocatalysis is illustrated in Fig. 1. The prochiral carbonyl group can be enantioselectively reduced to the corresponding chiral hydroxyl group by the microalgal intracellular oxidoreductase. The reduced cofactor, NAD(P)H, acting as the electron donor, is converted to $NAD(P)^+$. In photosynthesis, the oxidized $NAD(P)^+$ is directly regenerated to NAD(P)H through the light reaction utilizing solar energy.

Acetophenone

ACP is a preferred model substrate for simple ketone and aromatic ketone in asymmetric reduction reaction. The results of asymmetric reduction of ACP with various microalgae are given in Fig. 2, indicating that ACP can be reduced to *S*-PEA with excellent enantioselectivity. The *e.e.* of *S*-PEA was more than 97%. These results are similar to the asymmetric reduction of other aryl methyl ketones catalyzed by *Synechococcus* sp. PCC 7942 [17]. Among the two selected categories of microalgae, prokaryotic cyanophyta display higher catalytic activity than eukaryotic

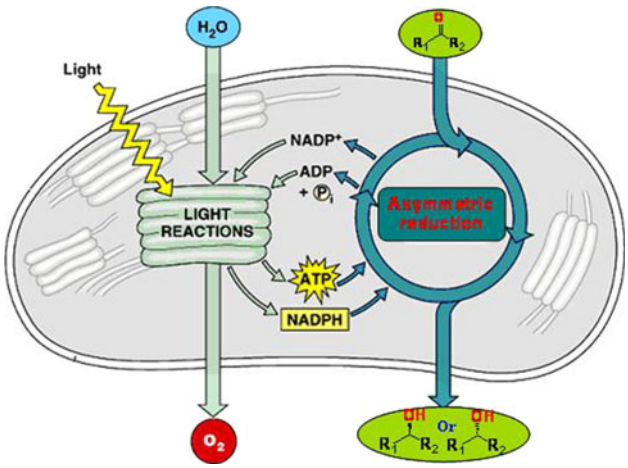

Fig. 1 Model scheme of the microalgal photo-biocatalytic asymmetric reduction reaction of prochiral ketones to chiral alcohols

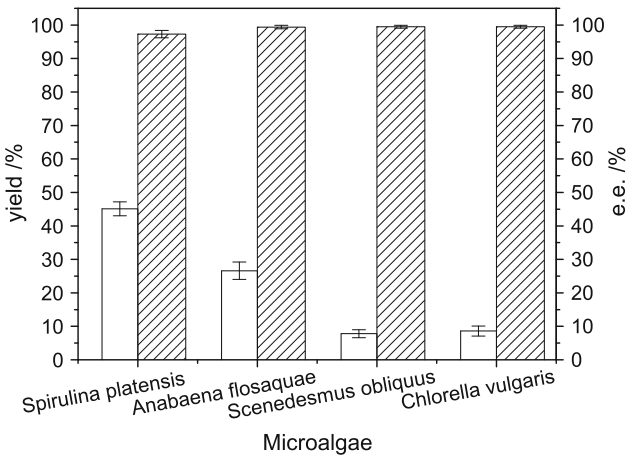

Fig. 2 Photo-biocatalytic asymmetric reduction of ACP by various microalgae. *Open rectangles* yield, *crossed rectangles e.e*

chlorophyta. Especially, *S. platensis* possesses the highest activity. The PEA chemical yield can reach up to 45% with this microalgae. This proves that cyanophyta are good biocatalysts for asymmetric reduction of aromatic ketone by photo-biocatalysis.

For bioconversion by active cells, substrate concentration is a key factor, since high substrate concentration will inhibit cell activity and catalytic activity. To explore this, the effect of substrate concentration on this reaction catalyzed by *S. platensis* and *A. flosaquae* was investigated. Due to the inherent toxicity of aromatic compound, the substrate concentration ranged between 3 and 8 mmol L^{-1}. As shown in Fig. 3, no remarkable effect of ACP concentration on enantioselectivity was observed. However, the effect of ACP concentration on reaction yield is very significant. The yield obviously decreases with increasing ACP concentration. The reason is that aromatic ketone is

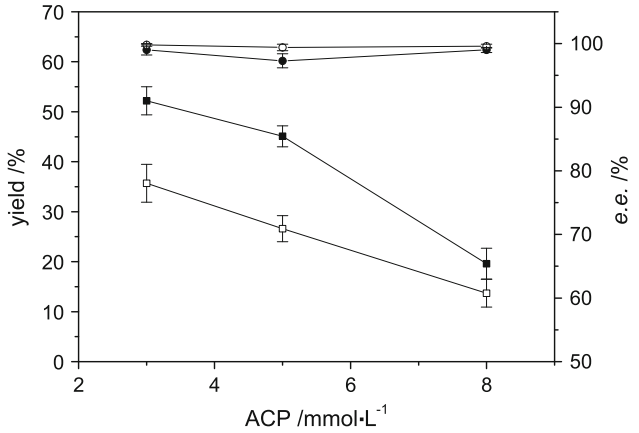

Fig. 3 Effect of ACP concentration on asymmetric reduction of ACP by microalgal photo-biocatalysis. *Filled squares* yield for *Spirulina platensis*, *open squares* yield for *Anabaena flosaquae*, *filled circles e.e.* for *Spirulina platensis*, *open circles e.e.* for *Anabaena flosaquae*

Table 1 Effect of illumination on asymmetric reduction of ACP by microalgae

Light	*Spirulina platensis*		*Anabaena flosaquae*	
	Yield (%)	*e.e.* (%)	Yield (%)	*e.e.* (%)
On	45.1 ± 2.1	97.3 ± 1.1	26.6 ± 2.6	99.4 ± 0.5
Off	2.7 ± 1.5	99.5 ± 0.4	2.1 ± 1.2	100.0 ± 0.0

noxious to the microalgae. Compared with the results from plant tissue catalyst [4], the microalgal tolerance to aromatic compound is lower than that of plant tissue.

To confirm the regeneration efficiency of cofactor NAD(P)H by the light reaction during photosynthesis, we investigated the effect of illumination on the microalgal photo-biocatalytic asymmetric reduction reaction. The reaction was conducted under 9,000 lux illumination or without illumination (in dark status) with 5 mmol L^{-1} ACP. The results are presented in Table 1. Exposed to illumination, *S. platensis* and *A. flosaquae* could catalyze the asymmetric reduction of ACP with good reaction activity. In contrast, without illumination, they practically lost the catalytic activity. In fact, the intracellular oxidoreductase that is responsible for the ACP asymmetric reduction reaction does not depend on light, but it does depend on reduced NAD(P)H. Therefore, it can be deduced that illumination is indispensable to cofactor regeneration.

Also, we investigated the effect of extra cosubstrate on the photo-biocatalytic asymmetric reduction reaction. Glucose and sodium citrate (0.5 g L^{-1}) were used as cosubstrates. The initial ACP concentration was 5 mmol L^{-1}. Results, presented in Table 2, prove that the tested microalgae do not need additional cosubstrates. This is quite different from other biocatalysts, such as microbial cells or plant tissue. However, the microalgal catalytic activity is

Table 2 Effect of cosubstrate on asymmetric reduction of ACP by microalgae

Cosubstrate	*Spirulina platensis*		*Anabaena flosaquae*	
	Yield (%)	*e.e.* (%)	Yield (%)	*e.e.* (%)
None	45.1 ± 2.1	97.3 ± 1.1	26.6 ± 2.6	99.4 ± 0.5
Glucose	61.5 ± 3.1	98.0 ± 0.7	37.6 ± 2.0	99.9 ± 0.1
Sodium citrate	52.4 ± 2.36	99.9 ± 0.1	24.3 ± 2.8	99.5 ± 0.4

Table 3 Asymmetric reduction of ACP catalyzed by various biocatalysts

Biocatalyst	Yield (%)	*e.e.* (%)	Conf.	Reference
Spirulina platensis	45.1	97.3	*S*	This study
Anabaena flosaquae	35.7	99	*S*	This study
Synechococcus sp. PCC 7942	3	96	*S*	[17]
Saccharomyces cerevisiae	24.5	98.0	*S*	[32]
Rhodotorula sp. AS2.2241	34.7	99.5	*S*	[19]
Rhizopus arrhizus	33.2	71.5	*S*	[24]
Trichothecium sp.	85.0	93.5	*R*	[14]
Malus pumila	40.9	81.5	*R*	[34]
Tessaria absinthioides	96[a]	98	*S*	[20]
Daucus carota	52[a]	98	*S*	[20]

[a] % Conversion

remarkably improved with addition of glucose as cosubstrate. This demonstrates that cyanophyta, like bacteria and fungi, can utilize carbohydrate to regenerate cofactors through respiratory metabolism. With additional cosubstrate, the NAD(P)H regeneration efficiency can be improved. This is the reason why carbohydrate cosubstrate can improve the reaction yield, providing a potential way to further improve the reaction yield.

For comparison, we summarize the published results of asymmetric reduction of ACP with commonly used biocatalysts, including bacteria, fungi, and plant tissues, in Table 3. Compared with these biocatalysts, the cyanophyta are attractive biocatalysts for the aromatic ketone asymmetric reduction reaction in terms of yield and *e.e.*

Ethyl acetoacetate

The yield and enantioselectivity of EAA asymmetric reduction catalyzed by the four microalgal species are shown in Fig. 4. The results indicate that EAA can be reduced to the corresponding *S*-form chiral alcohol,

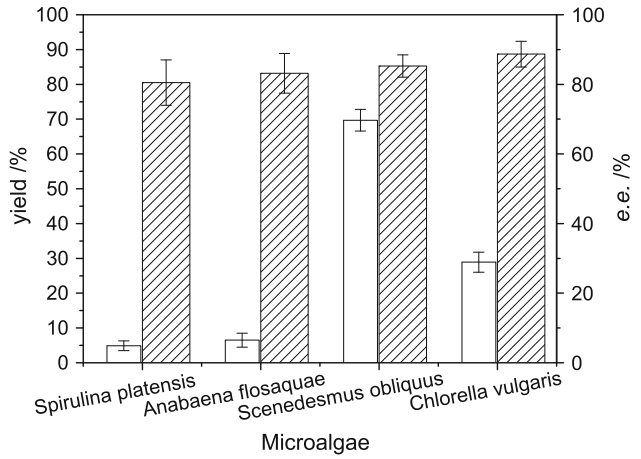

Fig. 4 Photo-biocatalytic asymmetric reduction of EAA by various microalgae. *Open rectangles* yield, *crossed rectangles e.e*

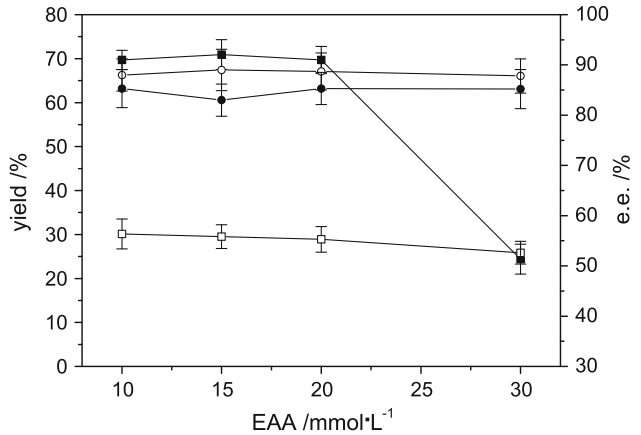

Fig. 5 Effect of EAA concentration on the asymmetric reduction of EAA by microalgal photo-biocatalysis. *Filled squares* yield for *Scenedesmus obliquus*, *open squares* yield for *Chlorella vulgaris*, *filled circles e.e.* for *Scenedesmus obliquus*, *open circles e.e.* for *Chlorella vulgaris*

S-EHB, with attractive yield and stereoselectivity. This proves that the β-ketoester can also be reduced to the corresponding chiral β-hydroxy esters by these microalgae. In contrast to the ACP asymmetric reduction reaction, eukaryotic chlorophyta are the preferred biocatalysts for the EAA asymmetric reduction reaction. Among the selected microalgae, *S. obliquus* is the most outstanding biocatalyst for the β-ketoester asymmetric reduction reaction. The yield and *e.e.* can reach about 70 and 85%, respectively.

Also, we investigated the effect of the EAA concentration on this reaction for the EAA concentration range between 10 and 30 mmol L^{-1}. The results are shown in Fig. 5. It can be seen that the enantioselectivity is not affected by the substrate concentration. However, the effect of EAA concentration on the yield is remarkable. Especially for *S. obliquus*, the yield sharply declined when the EAA concentration was more than 20 mmol L^{-1}.

Table 4 Effect of illumination on asymmetric reduction of EAA by microalgae

Light	*Scenedesmus obliquus*		*Chlorella vulgaris*	
	Yield (%)	*e.e.* (%)	Yield (%)	*e.e.* (%)
On	69.7 ± 3.1	85.3 ± 3.2	28.9 ± 2.9	88.7 ± 3.7
Off	2.2 ± 0.7	100.0 ± 0.0	4.2 ± 1.4	100.0 ± 0.0

Table 5 Effect of cosubstrate on asymmetric reduction of ACP by microalgae

Cosubstrate	*Scenedesmus obliquus*		*Chlorella vulgaris*	
	Yield (%)	*e.e.* (%)	Yield (%)	*e.e.* (%)
None	69.7 ± 3.1	85.3 ± 3.2	28.9 ± 2.9	88.7 ± 3.7
Glucose	83.5 ± 3.6	86.1 ± 4.7	34.5 ± 3.2	91.4 ± 2.9
Sodium citrate	75.4 ± 3.8	86.4 ± 3.7	29.3 ± 3.5	89.6 ± 3.5

The effect of illumination on the EAA reduction reaction was also investigated with 9,000 lux light intensity or without illumination. EAA concentration was 20 mmol L^{-1}. The results are given in Table 4. Similar to the asymmetric reduction of ACP by cyanophyta, illumination is also essential to the chlorophyta photo-biocatalysis process. This indicates that the NAD(P)H regeneration process in chlorophyta is similar to that in cyanophyta. In Table 4, the *e.e.* data ostensibly indicate that lack of illumination can remarkably improve the enantioselectivity. In fact, this is illusionary, because in the dark, the concentration of EHB (the product of EAA reduction) is very low, and especially the minor enantiomer, *R*-EHB, cannot be effectively determined by chiral HPLC. This causes the *e.e.* to become 100%.

The results for the effect of additional cosubstrate on the EAA reduction reaction are presented in Table 5. Glucose and sodium citrate, 0.5 g L^{-1}, was used as the cosubstrate, respectively. It was found that the additional carbohydrate could improve *S. obliquus* and *C. vulgaris* catalytic activity, providing evidence that the chlorophyta can also utilize carbohydrate to regenerate the cofactor, NAD(P)H, by respiratory metabolism. This is the same as for the ACP reduction reaction using cyanophyta photo-biocatalyst.

The results of EAA asymmetric reduction reaction catalyzed with commonly used biocatalyst are summarized in Table 6. Compared with the other biocatalysts, *S. obliquus* and *C. vulgaris* show attractive catalytic activity for EAA.

Table 6 Asymmetric reduction of ACP catalyzed by various biocatalysts

Biocatalyst	Yield (%)	*e.e.* (%)	Conf.	Reference
Scenedesmus obliquus	69.69	87.6	*S*	This study
Chlorella vulgaris	25.2	90.2	*S*	This study
Saccharomyces cerevisiae	90[a]	98	*S*	[15]
Pichia membranaefaciens Hansen	68.5	65.1	*R*	[8]
Paracoccus denitrificans	30	98.9	*R*	[18]
Daucus carota	58	95	*S*	[29]

[a] % Conversion

Conclusions

This work indicates that nonnatural prochiral ketones (aromatic ketones and β-ketoesters) can be reduced to corresponding chiral alcohols by prokaryotic cyanophyta and eukaryotic chlorophyta photo-biocatalytic processes with high enantioselectivity. For aromatic ketone, cyanophyta are eligible biocatalysts. On the contrary, chlorophyta are preferred biocatalysts for the β-ketoester asymmetric reduction reaction. Illumination is essential to the microalgal photo-biocatalysis to regenerate the cofactor, NAD(P)H. Similar to microbial cells, the microalgae can also regenerate NAD(P)H by utilizing carbohydrate through respiratory metabolism. For ACP asymmetric reduction, about 45% yield and 97% *e.e.* can be achieved through the *S. platensis* photo-biocatalytic process. In the case of EAA reduction, 70% yield and 90% *e.e.* can be achieved through the *S. obliquus* photo-biocatalytic process. This provides a green technique for chiral alcohol production.

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