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Asymmetric Reduction of Prochiral Ketones to Chiral Alcohol by Photo-biocatalysis with Microalgae

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***Abstract*—The photo-biocatalytic ability of eukaryotic microalgae and prokaryotic microalgae on nonnatural prochiral ketones to produce chiral alcohols was studied in this paper. The results proved that ethyl acetoacetate and acetophenone can be stereo-selectively reduced to the corresponding S-ethyl-3-hydroxybutyrate and S-1-phenylethanol by microalgal photo-biocatalysis with high enantioselectivity, respectively. *Scenedesmus obliquus* has the best photo-biocatalytic effect on ethyl acetoacetate among two eukaryotic microalgae, about 43.98% yield and 69.58% e.e. can be achieved, especially, the culture time is 6 days and light intensity is 9,000lux. *Spirulina platensis* is the best biocatalyst to acetophenone among four prokaryotic microalgae, about 52.65% yield and 99.40% e.e. can be achieved.**

***Keywords*—microalgae; asymmetric reduction; Photo-biocatalysis; Chiral alcohols**

I. INTRODUCTION

Because of the special biological activity, chiral substances have a great significance in many fields such as medicine, food, pesticides and fine chemicals. The chiral alcohols and chiral intermediates are the key buliding block to synthesize chiral products [1, 2]. The asymmetric reduction reaction by biocatalysis, especially by whole-cell biocatalysis, including yeast cells [3], other microorganisms [4, 5] and plant cells [6, 7] has become a research hotspot of chiral material production , due to the special biological stereoselective synthesis of chiral substances and its advantages such as outstanding enantioselectivity, mild reaction conditions and environmental friendliness, and regeneration of cofactor in situ in whole cells [8]. Microalgae are better photo-biocatalyst candidates for the asymmetric reduction reaction than other biocatalysis, due to the following merits: Firstly, they need lower b/s (the amount of biocatalyst / substrate amount), such as blue-green algae b/s is 0.3, while yeast b/s is up to 50-350. Secondly, they have higher stereoselectivity with broad substrate specificity, e.e. > 99% can be achieved by microalgae biocatalytsis. Thirdly, they have simpler operation and higher growth rate, and they are not easy to contamination. Lastly, microalgae catalytsis are more green, sustainable development and environmental compatibility, and can directly use solar energy to achieve coenzyme regeneration without other reducing substances (such as sugars and alcohol) as an energy and hydrogen donor [8]. When current energy and environmental issues have become the two key issues that hinder sustainable human development, microalgae produce biodiesel technology and microalgae carbon dioxide emission reduction technology are considered to be one of the most promising technologies to solve these two problems [9]. On this basis, combining with microalgae asymmetric reduction technology, the production of chiral substances in solving energy and environmental crisis to accelerate the transformation of economic development and new technologies will be more important significance.

There are a few reports regarding microalgal photo-biocatalytic asymmetric reduction reactions. *Synechococcus sp. PCC 7942* and *Synechosystis sp. 6803* (prokaryotic microalgae) were researched on the reduction of the carbonyl group in 3-acetylisoxazole derivatives to obtain the corresponding (S) -alcohols having a high enantioselectivity [10]. Also *Cyanidioschyzon merolae* (eukaryotic microalgae) was researched on the stereoinversion of ortho-, meta- and para-substituted fluoro, chloro, bromo and methyl 1-phenylethanols to gave the corresponding (S)-alcohols in high e.e. and high yield (95%, 91% ee) [11]. *Chlorella pyrenoidosa* Chick catalyzed the reduction of methyl 3-oxopentanoate (Me 3-oxoPen) to the corresponding (S)-(+)-3-hydroxy ester in 60% enantiomeric excess (e.e.) [12]. The above studies proved that prokaryotic microalgae and eukaryotic microalgae both had applications in asymmetric reduction, but there exhibited different catalytic activity on different type of reaction substrate. Therefore, it is necessary to identify the ability of photo-biocatalytsis proachiral ketones with microalgae. The objective of this study is to explore the possibility of asymmetric reduction of aromatic ketones and β-ketoesters by microalgal photo-biocatalytic reaction. Six microalgae, *Scenedesmus obliquus*, *Chlorella vulgaris*, *Spirulina platensis*, *Anabaena flosaquae*, *Synechocystis sp. PCC 6803* and *Synechococcus sp. PCC 7942* were chosen as photo-biocatalysts. The first two are eukaryotic microalgae, and the others are prokaryotic microalgae. Acetophenone and ethyl acetoacetate are chosen as model substrates for aromatic ketones and β-ketoesters, respectively, which are widely applied in practical asymmetric synthesis. And, we also explored the conditions to get higher chemical yield and enantiomeric excess to photo-biocatalytsis ethyl acetoacetate and acetophenone to the corresponding chiral alcohols.

II. EXPERIMENT

A. Materials

Acetoacetate(ACP) and benzaldehyde were purchased from Sinopharm Chemical Reagent Co., Ltd. (China) as analytical-grade reagents. R- and S-1-phenylethanol (S-PEA), ethy lacetoacetate (EAA), S-ethyl-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, n-hexane and other reagents were of analytical grade and commercially available.

B. Microalgae and Cuture Medium

S. platensis, *A. flosaquae*, *S. obliquus*, *C. vulgaris*, *Synechocystis sp. PCC 6803* and *Synechococcus sp. PCC 7942* 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: modified soil extract (SE) medium medium was used for *S. obliquus* culture [13], and BG-11 was used for *S. platensis*, *A. flosaquae*, *C. vulgaris*, *Synechocystis sp. PCC 6803* and *Synechococcus sp. PCC 7942* culture [10].

C. Experiment Process

1) Microalgae culture: They were incubated at 28°C with 9,000 lux illumination on the photo-bioreactor surface provided by a continuous cool white fluorescent light. A 5% CO2 gas (v/v, mixed with air), provided by a gas cylinder, was aerated from the photo-bioreactor bottom at rate of 0.1 v/v min^{-1} (volume gas per volume broth per minute).

2) Catalytic reaction: The reaction was carried out under 28°C for a certain period to obtain a favorable conversion degreen in the light incubator.

3) Extraction: After a certain time of reaction, EAA and ACP reaction mixture were extracted with isovolumetric n-hexane and ethyl acetate, respectively, the organic phase was dried by anhydrous Mg_2SO_4.

D. Analysis.

The concentrations of EAA and EHB were determined by gas chromatography (GC, model 6890; Agilent Technologies Co., Ltd.) equipped with a flame ionization detector and achiral Cyclodex-B capillary column(30 m, 0.25 mm, 0.25 mm), using n-octanol as internal standard. The split ratio was 1:20. The temperature for the injector and detector were kept at 250° C and 300° C, respectively. The column temperature was held at 80 °C for 3min and was increased to 120 °C at a rate of 8 °C/min, and then kept constant for 10 min. The carrier gas was nitrogen and its flow rate in the column was 1.2 ml/min. An internal standard method was used for the calculations. The retention times for EAA and EHB were 11.532min and 11.997min in Fig. 1(a), respectively.

The optical purity of the formed EHB was 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 (49/1, v/v) as mobile phase, flow rate of 1.5 mL/min, ambient column temperature, and ultraviolet (UV) detection set at 215 nm. The retention times for R-EHB and S-EHB were 6.609min and 9.241min in Fig. 1(b), respectively.

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 N2 carrier gas with 1.5 mL/min flow rate, splitting ratio of 10:1, flame ionization detector (FID), and the following oven temperature program: 90 °C for 4 min, increasing from 90 °C to 115 °C at rate of 1 °C /min, and holding at 115 °C for 1 min. The retention times for ACP, R- and S-PEA were 19.736min, 25.632 and 26.362min in Fig. 1(c), respectively.

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

$$\text{Yeild} = C_P / C_O \times 100 \quad (1)$$

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

where C_O 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.

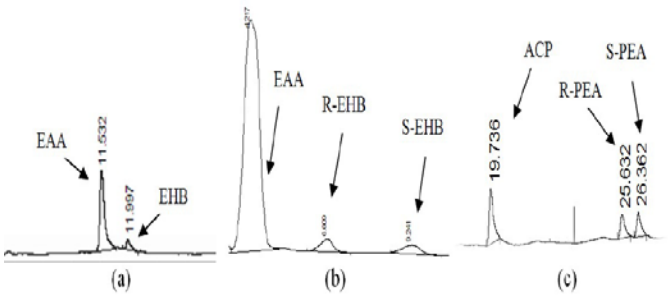

Fig. 1. a) GC analysis on EAA. b) HPLC analysis on R-(S-) EHB. c) GC analysis on ACP and R-(S-) PEA.

III. RESULTS AND DISCUSSION

The photo-biocatalytic ability of eukaryotic microalgae and prokaryotic microalgae on nonnatural prochiral ketones (EAA and ACP) to produce corresponding chiral alcohols (EHB and PEA) was investigated. Six microalgae, *Scenedesmus obliquus*, *Chlorella vulgaris*, *Spirulina platensis*, *Anabaena flosaquae*, *Synechocystis sp. PCC 6803* and *Synechococcus sp. PCC 7942* were chosen as biocatalysts. Acetophenone and ethyl acetoacetate are chosen as model substrates for aromatic ketones and β-ketoesters, respectively. 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)H. In photosynthesis, the oxidized NAD(P)H is directly regenerated to NAD(P)H through the light reaction utilizing solar energy. Thus, we explored conditions to get

higher chemical yield and enantiomeric excess to photo-biocatalytsis EAA and ACP to the corresponding chiral alcohols.

A. *Reduction Reaction Catalyzed by Various Microalgae*

In order to identify the ability of photo-biocatalytsis EAA with microalgae, we compared four microalgae, two eukaryotic microalgae and two prokaryotic microalgae. The study founded that two eukaryotic microalgae, *C. vulgaris* and *S. obliquus* had a high asymmetric reactivity, but two prokaryotic microalgae, *A. flosaquae*, *S. platensis* had a poor asymmetric reactivity. The data are not given, because we have discussed before[14]. The analysis showed that the catalytic ability of *C. vulgaris* to ethyl acetoacetate was very unstable, no matter what add a condition to increase the yield, the enantiomeric excess decreased, and vice versa. However, *S. obliquus* showed good ability, when culture time was 6 days, light intensity was 9,000lux , yield could reached up to 43.98% and e.e. reach up to 69.58%, as given in Fig. 2. Comprehensive comparison, *S. obliquus* is the best microalgae to asymmetric catalyze EAA, and will be studied in the following experiments.

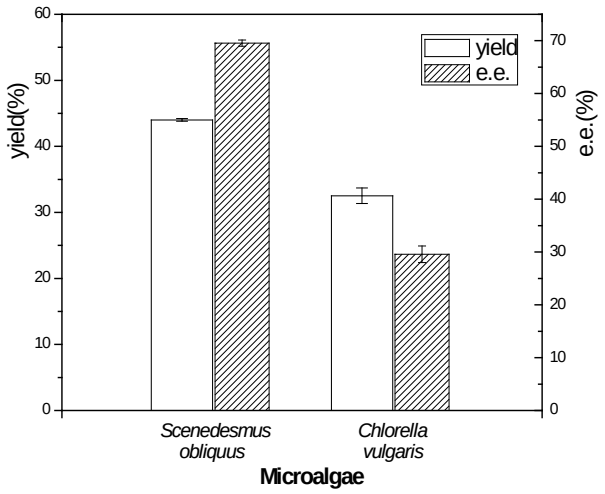

Fig. 2. Photo-biocatalytic asymmetric reduction of EAA by various microalgae.

In order to identify the ability of photo-biocatalytsis ACP with microalgae, we compared four prokaryotic without using eukaryotic microalgae, because they were researched to have a poor photo-biocatalytic ability [14]. As showed in Fig. 3, the e.e. of four microalgae reached up to the percentage almostly, while the yield had obvious differences.The yield of *S. platensis* was one of the highest, reached up to 52.66%. Comprehensive comparison, *S. platensis* is the best microalgae to asymmetric catalyze ACP, and will be studied in the following experiments, too.

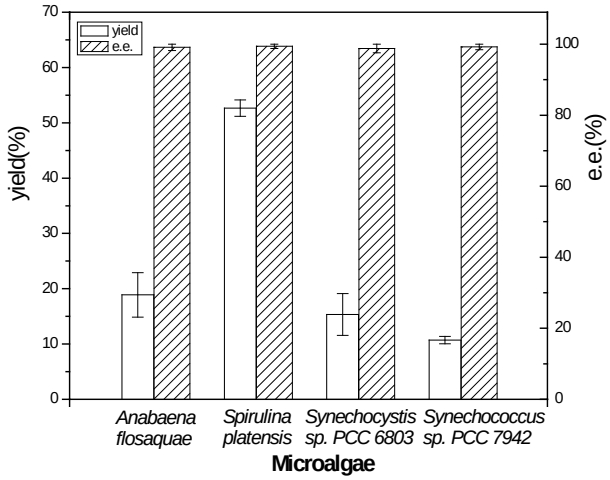

Fig. 3. Photo-biocatalytic asymmetric reduction of ACP by various microalgae.

B. *Effect of Culture Time.*

Microalgae growth curve divids into lag phase, logarithmic phase, stability phase and decline phase like bacteria. In the logarithmic phase, the large number of cells, high metabolic capacity, strong photosynthesis and high efficiency of coenzyme reproducing are conducive reductase to catalytsis substrate. So, we have researched the effect of culture time on photo-biocatalysis with microalgae. In the culture of 3 days, 6 days stood for in the logarithmic phase, 9 days stood for stationary phase. As showed in Fig. 4, there exhibited different catalytic activity to EAA. When the culture time was 6 days, which the concentrations of microalgae were high, the yield of *C. vulgaris* and *S. obliquus* reached up the maximum, while the e.e had a difference due to the catalytic activity of R-reductase and S-reductase were changed differently [15]. And as showed in Fig. 5, there exhibiedt different catalytic activity to ACP. When the culture time was 6 days, the yield of *Synechocystis sp. PCC 6803* and *Anabaena flosaquae* reached up the maximum, reached to 56.2% and 58.5% , higher than the other culture time, obviously. And the e.e almost closed to the percentage (100%). Therefore, the cells of logarithmic phase are conducive to catalytic reaction, also can shorten the reaction time. And the microalgae of logarithmic phase have a higher resistance to high concentration of prochiral substrate. So it is a huge research value to future industrial production of chiral substance.

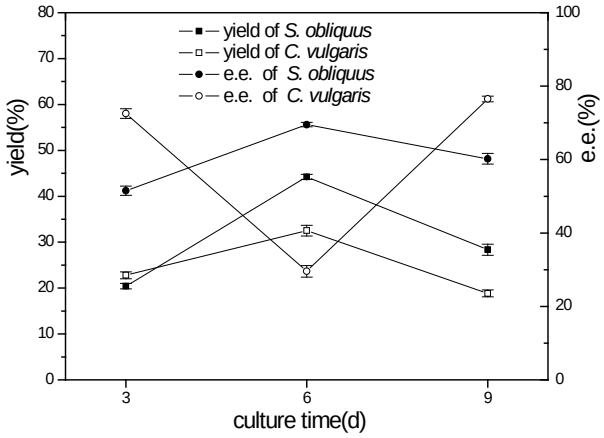

Fig. 4. Effect of reaction time on the asymmetric reduction of EAA by microalgal photo-biocatalysis.

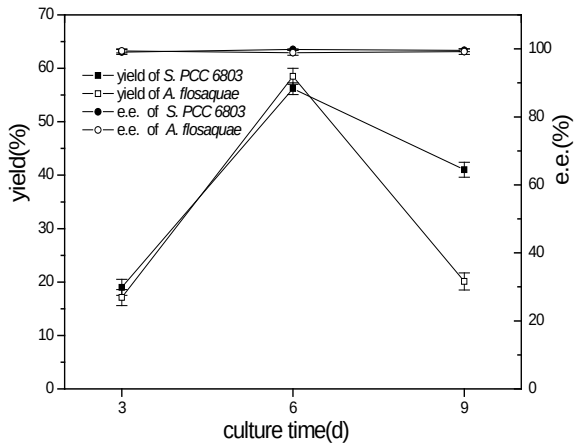

Fig. 5. Effect of reaction time on the asymmetric reduction of ACP by microalgal photo-biocatalysis.

C. Effect of Light Intensity.

In light conditions, microalgae can generate NADPH with photosynthesis, thus can asymmetric catalytsis prochiral ketones to corresponding chiral alcohols. NADPH is produced in the light reaction stage and is oxidized in the dark reaction stage. In light reaction stage, for bright light, PS II absorb reducing hydrogen more than PS I and to restore the hydrogen is faster than to oxidize the hydrogen, thus it is good for the accumulation of NADPH. On the contrary, for low light, PS I oxidize reducing hydrogen faster than PS II restore the hydrogen, it is not conducive to the accumulation of NADPH. So, stronger light intensity can accumulate the concentration of NADPH. However, light is the final activator for RuBP in dark reaction, excessive light intensity can also promote the dark reaction, this is not conducive to the accumulation of NADPH [16]. Therefor, a appropriate illumination intensity is essential to improve the asymmetric reduction of microalgae. As showed in Table 1, when the light intensity was 9,000lux , yield and e.e. of *S. obliquus* reached up to 43.98% and 69.58%, respectively, higher than 4,500lux. But, *C. vulgaris* had a poor result with e.e., while yield increased obviously. The reason is that the light intensity can also change the R- and S- enzymes activity, the R-enzymes activity was inhibited and S-enzymes activity increased [17, 18].

TABLE I. EFFECT OF LIGHT INTENSITY ON ASYMMETRIC REDUCTION OF EAA BY MICROALGAE

Light intensity [lux]	*Scenedesmus obliquus*		*Chlorella vulgaris*	
	Yield[%]	e.e. [%]	Yield[%]	e.e. [%]
0	27.2±0.7	0.0±0.0	0.0±0.0	0.0±0.0
4500	35.4±0.6	23.7±0.7	43.4±0.6	29.3±1.2
9000	44.0±0.2	69.5±0.6	18.8±0.7	76.5±0.8

IV. CONCLUSIONS

In the catalytic reaction of ethyl acetoacetate, eukaryotic microalgae have higher catalytic activity than prokaryotic microalgae, however, in the catalytic reaction of acetophenone, prokaryotic microalgae have higher catalytic activity than eukaryotic microalgae. Culture time and intensity of illumination have effect on the photo-biocatalysis with microalgae. The appropriate culture time and the appropriate intensity of illumination are conducive to increase the chemical yield and enantiomeric excess. This study will be the foundation for later study.

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