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Stereocontrolled Reduction of α- and β-Keto Esters with Micro Green Algae, *Chlorella* Strains

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The stereocontrolled reduction of α- and β-keto esters using micro green algae was accomplished by a combination of the cultivation method and the introduction of an additive. The reduction of ethyl pyruvate and ethyl benzoylformate by the photoautotrophically cultivated *Chlorella sorokiniana* gave the corresponding alcohol in high e.e. (>99% e.e. (*S*) and >99% e.e. (*R*), respectively). In the presence of glucose as an additive, the reduction of ethyl 3-methyl-2-oxobutanoate by the heterotrophically cultivated *C. sorokiniana* afforded the corresponding (*R*)-alcohol. On the other hand, the reduction in the presence of ethyl propionate gave the (*S*)-alcohol. Ethyl 2-methyl-3-oxobutanoate was reduced in the presence of glycerol by the photoautotrophically cultivated *C. sorokiniana* or the heterotrophically cultivated *C. sorokiniana* to the corresponding *syn*-(2*R*,3*S*)-hydroxy ester with high diastereo- and enantiomeric excess (e.e.). Some additives altered the stereochemical course in the reduction of α- and β-keto esters.

Key words: *Chlorella*; asymmetric reduction; keto ester; heterotrophic cultivation; photoautotrophic cultivation

Optically active alcohols are useful compounds in organic chemistry and are used as synthetic starting molecules for other chiral compounds. For example, because of their bifunctional properties, the optically pure hydroxy esters are useful building blocks for the synthesis of various biologically active substances.[1–5] For the preparation of such chiral alcohols, biotransformation has been widely studied.[6–9] In particular, yeast and fungi have been often used for the stereoselective reduction of prochiral ketones to obtain optically active alcohols, such as α- and β-hydroxy esters.[5,10,11]

To date, several studies concerning the reduction of keto esters with other microorganisms (except yeast and fungi) have been reported.[5,12] However, little information is known about the reduction of carbonyl compounds using such microorganisms as biocatalysts. Recently, we described how aerobic thermophilic bacteria such as *Bacillus stearothermophilus*,[13] *Pseudonocardia thermophila*,[14] and *Streptomyces thermocyaneoviolaceus*[15] had high reducing abilities toward several keto esters and produced the corresponding chiral hydroxy esters. In this paper, we report the stereocontrolled reduction of α- and β-keto esters by some *Chlorella* strains as new biocatalysts. (Fig. 1)

Materials and Methods

Instruments. Gas chromatography was done using GL Science GC-353 (DB-Wax, J&W Scientific, USA, 0.25 mm × 30 m; CP-Chirasil-DEX CB, Chrompack, Netherlands, 0.25 mm × 25 m) gas chromatographs.

Materials. Ethyl 2-methyl-3-oxobutanoate (**1**) and ethyl pyruvate (**3a**) and were purchased from Wako Pure Chemical Industries, Ltd., Japan. Ethyl 3-methyl-2-oxobutanoate (**3f**) was purchased from Aldrich, USA. Ethyl benzoylformate (**3g**) was obtained

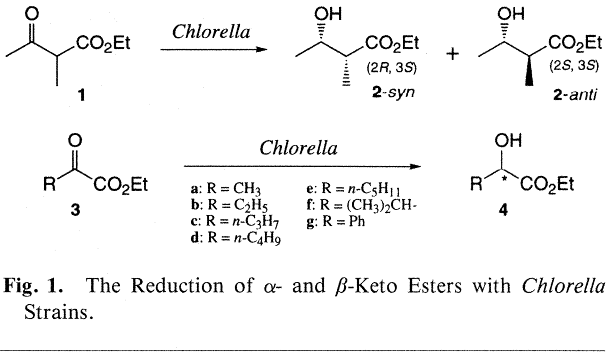

Fig. 1. The Reduction of α- and β-Keto Esters with *Chlorella* Strains.

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from Tokyo Kasei Kogyo, Japan. Ethyl 2-oxobutanoate (**3b**), ethyl 2-oxopentanoate (**3c**), ethyl 2-oxohexanoate (**3d**), ethyl 2-oxoheptanoate (**3e**), and α-hydroxy esters (**4a–g**) were prepared according to the procedures in the literature.[10]

Chlorella strains and culture conditions. Chlorella vulgaris (IAM C-27, C-135, C-207), *Chlorella fusca* (IAM C-30), *Chlorella saccharophila* (IAM C-169), and *Chlorella protothecoides* (IAM C-206) were obtained from the IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo. *Chlorella sorokiniana* SAG 211-8k (one of the thermophilic strains) was kindly donated by Dr. Isao Maruyama, Research Laboratories, Chlorella Industries, Co., Ltd., Fukuoka, Japan. The *Chlorella* strains were maintained at 15°C in the following synthetic media (Media A ~ E) solidified 1.5% agar. Medium-A (for IAM C-27) is comprised of 1.25 g KNO_3, 1.25 g $MgSO_4 \cdot 7H_2O$, 1.25 g KH_2PO_4, 1.0 ml of Fe solution, and 1.0 ml of A_5 solution per 1 liter of distilled water (pH 6.0). Fe solution is one of the microelements solutions: $FeSO_4 \cdot 7H_2O$ (1.0 g), and conc. H_2SO_4 (2 drops) were dissolved in 1 liter of distilled water. A_5 solution is also one of the microelements solutions: H_3BO_3 (2.86 g), $MnSO_4 \cdot 7H_2O$ (2.5 g), $ZnSO_4 \cdot 7H_2O$ (0.222 g), $CuSO_4 \cdot 5H_2O$ (79 mg), and Na_2MoO_4 (21 mg) were dissolved in 1 liter of distilled water. Medium-B (for IAM C-30 and C-169) is comprised of 0.15 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.1 g KNO_3, 40 mg $MgSO_4 \cdot 7H_2O$, 50 mg β-glycerophosphate disodium salt, 10 μg vitamin B_1, 0.1 μg vitamin B_{12}, 0.1 μg biotin, 0.5 g Tris buffer, and 3.0 ml of P IV metals solution per 1 liter of distilled water (pH 7.5). P IV metals solution is one of the microelements solutions: $FeCl_3 \cdot 6H_2O$ (0.194 g), $MnCl_2 \cdot 4H_2O$ (82 mg), $ZnCl_2$ (10 mg), $CoCl_2 \cdot 6H_2O$ (4 mg), Na_2MoO_4 (8 mg), and EDTA disodium salt (1.5 g) were dissolved in 1 liter of distilled water. Medium-C (for IAM C-135) is comprised of 1.0 g KNO_3, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.25 g K_2HPO_4, 0.1 g NaCl, 10 mg $CaCl_2 \cdot 2H_2O$, 1.0 ml of Fe solution, and 1.0 ml of A_5 solution per 1 liter of distilled water (pH 8.0). Medium-D (for IAM C-206, C-207, and SAG 211-8k) is comprised of 1.0 g KNO_3, 75 mg $MgSO_4 \cdot 7H_2O$, 75 mg K_2HPO_4, 175 mg KH_2PO_4, 25 mg NaCl, 10 mg $CaCl_2 \cdot 2H_2O$, 1.0 ml of Fe solution, 1.0 g proteose peptone, and 1.0 ml of A_5 solution per 1 liter of distilled water (pH 6.0). The *Chlorella* strains were photoautotrophically grown in each maintained medium (800 ml) for 8 d at 25°C (*C. sorokiniana* was grown at 30°C) with aerobic shaking in baffled 2-liter flasks with illumination by white fluorescent light (1000 lx, one side). In the heterotrophic cultivation, the *Chlorella* strains were grown for 4 d at 25°C (*C. sorokiniana* was grown at 30°C) with aerobic shaking in baffled 2-liter flasks with illumination by white fluorescent light (1000 lx, one side) in the glucose-rich medium (Medium-E, 800 ml). Medium-E is comprised of 1.5 g urea, 60 mg $MgSO_4 \cdot 7H_2O$, 1.2 g KH_2PO_4, 15 mg EDTA Na-Fe salt, 20 g glucose, and 1.0 ml of A_5 solution per 1 liter of distilled water (pH 6.0).

Reduction of keto esters using Chlorella strains. The algal cells photoautotrophically cultivated in each maintained medium or heterotrophically cultured in Medium-E were harvested by centrifugation at 8,000 × *g* for 20 min at 4°C, and then the harvested cells was washed with saline. The washed cells (0.5 g) were resuspended in a large test tube (ϕ30 mm × 200 mm) containing 20 ml of saline, and then the substrate (0.15 mmol) was added and incubated at 30°C with aerobic shaking in the light (1000 lx, one side). A portion of the reaction mixtures were filtered using an Extrelut® (Merck, Germany) short column and then concentrated under reduced pressure after extraction with diethyl ether.

Measurement of conversion, enantiomeric excess and diastereoselectivity. The conversions of products (**2, 4a–g**) were measured using a GLC with a capillary DB-WAX column (0.25 mm × 30 m, 110 ~ 180°C). The diastereoselectivity (*syn*/*anti* ratio) of **2** was also measured using a GLC with a DB-WAX column (120°C). The enantiomeric excesses (e.e.) of the products were measured using a GLC equipped with an optically active capillary CP-Chirasil-DEX CB, 0.25 mm × 25 m, 70 ~ 130°C) column (**2**, **4a–e**, and **4g**) and Chiraldex G-TA (0.25 mm × 40 m, 90°C) column (**4f**). The absolute configurations of the α-hydroxy esters **4a–g** were identified by comparing their retention time with those of authentic samples.[10] The absolute configuration of the isomer corresponding to each peak of **2** obtained from the reduction of the substrate by $NaBH_4$ (composed of all four isomers) was identified by comparing its retention time with those of the authentic samples prepared by methylation of racemic and ethyl (*S*)-3-hydroxybutanoate,[16] and by the reduction of the substrate by *Chlorella* strains.

Results and Discussion

Stereoselective reduction by Chlorella strains

Seven selected *Chlorella* strains were examined for their α- and β-keto ester reducing activities (Fig. 1). All of the *Chlorella* strains tested had the ability to reduce ethyl 2-methyl-3-oxobutanoate (**1**) and the α-keto esters (**3a–g**) as shown in Tables 1 and 2. The algal reduction of **1** with the heterotrophically cultivated *Chlorella* strains tended to slightly increase the conversion. The reduction **1** by *C. saccharophila* and *C. protothecoides* gave the corresponding *anti*-hydroxy ester. For example, the substrate was reduced to the *anti*-hydroxy ester with high diastereoselectivity (*syn*/*anti* = 7:93) by the heter-

Table 1. Reduction of Ethyl 2-Methyl-3-oxobutanoate (**1**) by Seven *Chlorella* Strains[a]

Strain	IAM or SAG No.	Photoautotrophic cultivation[b] Medium	Conv.[d] (%)	*Syn*/*Anti*[d]	e.e. (%)[e] *Syn*-(2*R*,3*S*)	*Anti*-(2*S*,3*S*)	Heterotrophic cultivation[c] Medium	Conv.[d] (%)	*Syn*/*Anti*[d]	e.e. (%)[e] *Syn*-(2*R*,3*S*)	*Anti*-(2*S*,3*S*)
C. vulgaris	IAM C-27	A	41	64/36	59	20	E	39	60/40	70	19
C. fusca	IAM C-30	B	55	72/28	88	75	E	66	71/29	89	81
C. vulgaris	IAM C-135	C	38	73/27	68	79	E	49	69/31	82	66
C. saccharophila	IAM C-169	B	54	28/72	72	69	E	67	36/64	84	78
C. protothecoides	IAM C-206	D	57	16/84	90	92	E	65	7/93	98	96
C. vulgaris	IAM C-207	D	69	60/40	83	70	E	74	63/37	78	71
C. sorokiniana	SAG 211-8k	D	98	>99/<1	>99	—[f]	E	99	93/7	91	99

[a] Saline (20 ml) and ethyl 2-methyl-3-oxobutanoate (**1**) (7.5 mM) were added to the wet cells (0.5 g) and the reaction mixtures were incubated at 30°C for 72 h under light (1000 lx).

[b] Photoautotrophic cultivation: *Chlorella* cells were grown in each maintained medium (Media A ~ D) under light (1000 lx).

[c] Heterotrophic cultivation: *Chlorella* cells were grown in the glucose-rich medium (Medium-E) under light (1000 lx).

[d] The ratio of *syn*/*anti* and the conversion were measured by GLC with a capillary column DB-WAX (0.25 mm × 30 m).

[e] The e.e. (%) and configuration were measured by GLC with an optically active capillary column CP-Chirasil-DEX CB (0.25 mm × 25 m).

[f] Not determined (not detected of the peak from the *anti*-hydroxy ester in GC analysis).

Table 2. Reduction of α-Keto Esters by *Chlorella sorokiniana*[a]

Product	Photoautotrophic cultivation[b] Conv.[d] (%)	e.e.[e] (%)	(*R*/*S*)	Heterotrophic cultivation[c] Conv.[d] (%)	e.e.[e] (%)	(*R*/*S*)
4a	89	>99	*S*	>99	59	*S*
4b	30	79	*S*	40	68	*S*
4c	55	40	*S*	81	52	*S*
4d	33	50	*S*	44	65	*S*
4e	15	55	*S*	24	64	*S*
4f	86	60	*R*	>99	17	*R*
4g	98	>99	*R*	>99	23	*R*

[a] Saline (20 ml) and α-keto ester (**3a–g**) (7.5 mM) were added to the wet cells (0.5 g) and the reaction mixtures were incubated at 30°C for 72 h under light (1000 lx).

[b] *Chlorella* cells were grown in Medium-D under light (1000 lx).

[c] *Chlorella* cells were grown in Medium-E under light (1000 lx).

[d] The conversion was measured by GLC with a capillary column DB-WAX (0.25 mm × 30 m).

[e] The e.e. (%) and configuration were measured by GLC with an optically active capillary column CP-Chirasil-DEX CB (0.25 mm × 25 m).

otrophically cultivated *C. protothecoides*. In the *syn*/*anti* ratio, the reduction by *C. vulgaris*, *C. fusca*, and *C. sorokiniana* preferentially gave the corresponding *syn*-hydroxy ester. In particular, the reduction by the photoautotrophically cultivated *C. sorokiniana* afforded the *syn*-hydroxy ester with excellent diastereo-(*syn*>99%) and enantioselectivity (>99% e.e.).

The reduction of α-keto esters by *Chlorella sorokiniana* that showed the best result in the reduction of ethyl 2-methyl-3-oxobutanoate was investigated (see Table 2). The reduction of **3a–g** with the *C. sorokiniana* heterotrophically cultivated also tended to increase the conversion, however, the enantioselectivity of the products was low (below 68% e.e.). For the reduction of **3a** and **3g**, the photoautotrophic cultivated *C. sorokiniana* afforded the corresponding α-hydroxy ester (*S*)-**4a** and (*R*)-**4g** in >99% e.e., respectively.

The *Chlorella* strain was found to be an available biocatalyst for the stereocontrolled reduction of α- and β-keto esters. It is worth noting that, in the algal reduction, the stereochemistry of the hydroxy esters produced could be controlled by changing the growth conditions (photoautotrophic or heterotrophic cultivation). It is suggested that these results are probably due to the differences in varieties and contents of the enzymes expressed in the photoautotrophic cultivated algal cells on the comparison with the those heterotrophically cultivated.

Effects of additives on stereoselectivity

It was reported that the stereoselectivity was improved by the introduction of additives in the yeast reduction of keto esters.[11] For the reduction of three α-keto esters (**3a**, **3f**, and **3g**) by *C. sorokiniana*, the effects of additives on the conversion and enantioselectivity of the produced α-hydroxy esters were investigated (see Table 3). The conversion of the reduction from **3a** (ethyl pyruvate) to **4a** in the presence of glycerol, glucose, and amino acids was a high ratio (>99%), however, the enantioselectivity of the product **4a** was a low value (43-56% e.e.). The reduction of **3f** (ethyl 3-methyl-2-oxobutanoate) with glucose gave the corresponding (*R*)-hydroxy alcohol (**4f**) with 91% e.e. On the other hand, the reduction with ethyl propionate gave the (*S*)-hydroxy alcohol with 96% e.e. This result shows that the changing of additives alters the stereoselectivity of the product for the algal reduction of **3f**. The introduction of glucose might be promote the coenzyme production for the (*R*)-hydroxy ester producing enzyme(s), while the (*R*)-enzyme would be inhibited by the addition of ethyl propionate. No additives we tried increased the conversion and enantioselectivity of the product in the case of the reduction of ethyl benzoylformate (**4g**).

Table 3. Effects of Additives on the Reduction of α-Keto Esters[a,b]

Additives	4a Conv.[c] (%)	4a e.e.[d] (%)	4f Conv.[c] (%)	4f e.e.[d] (%)	4g Conv.[c] (%)	4g e.e.[d] (%)
No additive	>99	59 (*S*)	>99	17 (*R*)	>99	23 (*R*)
Glycerol	>99	54 (*S*)	>99	49 (*S*)	54	4 (*R*)
Glucose	>99	55 (*S*)	70	91 (*R*)	98	75 (*R*)
Glycine	>99	43 (*S*)	>99	27 (*S*)	20	12 (*R*)
L-Alanine	>99	56 (*S*)	>99	22 (*S*)	29	15 (*S*)
Chloroacetone	2	74 (*S*)	24	34 (*S*)	40	25 (*R*)
Ethyl propionate	57	57 (*S*)	26	96 (*S*)	81	62 (*R*)
Allyl alcohol	78	77 (*S*)	50	51 (*S*)	53	1 (*R*)
3-Buten-2-one	31	23 (*S*)	8	30 (*S*)	15	16 (*R*)

[a] Saline (20 ml), α-keto ester (**3a**, **f**, **g**) (7.5 mM), and additive (75 mM) were added to the wet cells (0.5 g) and the reaction mixtures were incubated at 30°C for 72 h under light (1000 lx).
[b] *Chlorella* cells were grown in Medium-E (heterotrophic ciltivation) under light (1000 lx).
[c] The conversion was measured by GLC with a capillary column.
[d] The e.e. and configuration were measured by GLC with an optically active capillary column.

Table 4. Effects of Additives on the Reduction of β-Keto Esters[a,b]

Additives	Conv.[c] (%)	*Syn*/*Anti*[c]	e.e. (%)[d] *Syn*-(2*R*,3*S*)	e.e. (%)[d] *Anti*-(2*S*,3*S*)
No additive	83	92/8	>99	18
Glycerol	94	>99/<1	>99	>99
Glucose	>99	88/12	>99	98
Glycine	>99	86/14	96	40
L-Alanine	93	87/13	98	49
Chloroacetone	2	51/49	>99	>99
Ethyl propionate	1	71/29	>99	>99
Allyl alcohol	3	69/31	>99	36
3-Buten-2-one	7	82/18	>99	16

[a] Saline (20 ml), β-keto ester (**1a**) (7.5 mM), and additive (75 mM) were added to the wet cells (0.5 g) and the reaction mixtures were incubated at 30°C for 72 h under light (1000 lx).
[b] *Chlorella* cells were grown in Medium-E (heterotrophic ciltivation) under light (1000 lx).
[c] The conversion and *syn*/*anti* ratio were measured by GLC with a capillary column.
[d] The e.e. and configuration were measured by GLC with an optically active capillary column.

Ethyl 2-methylacetoacetate (**1**) was reduced by *C. sorokiniana* with glucose or amino acid to the corresponding β-hydroxy ester with a high conversion ratio, however, the diastereoselectivity was a low value. The algal reduction of **1** with glycerol afforded the *syn*-(2*R*, 3*S*)-hydroxy ester with high conversion in excellent diastereo-(*syn*>99%) and enantioselectivity (>99% e.e.) as shown in Table 4. The use of other additives such as chloroacetone and allyl alcohol during the reduction caused a decrease in the conversion ratio.

The additives such as 3-butene-2-one (methyl vinyl ketone), ethyl propionate, and allyl alcohol acted as a inhibitor for the enzyme(s) which produces one (*R*- or *S*-) enantiomer.[17] We considered that the increase of reduced coenzyme (maybe NAD(P)H) by the oxidative degradation of the nutrious additives (such as glucose, glycerol, and amino acids) in the algal cells would probably accelerate the stereoselective reduction of α-keto esters to the corresponding optically pure alcohols as we described previously.[13,14]

To gain insight into the mechanistic interpretation of the algal reduction, further detailed studies including purification of the oxidoreductases, which contribute to the stereoselective reduction of α- and β-keto esters, are currently under investigation.

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