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Journal of Molecular Catalysis B: Enzymatic 23 (2003) 171–189
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Review

Stereoselective reduction of keto esters: thermophilic bacteria and microalgae as new biocatalysts

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Received 7 February 2003; accepted 25 April 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

This review covers the possibility of aerobic thermophilic bacteria (*Bacillus* strains and thermophilic actinomycetes) and microalgae (*Chlorella* strains and marine algae) as new biocatalysts for the stereoselective reduction of α- and β-keto esters. The mechanistic interpretation of the reduction by a thermophilic actinomycete is also delineated.

Keywords: Thermophilic bacteria; *Bacillus* strains; Thermophilic actinomycetes; *Chlorella* strains; Marine algae; Keto ester; Stereoselective reduction

1. Introduction

The biological activities of optically active compounds such as agrochemicals, pharmaceuticals, and flavors often depend upon the configuration of their chiral center(s) [1,2]. Therefore, the stereo- and regioselective synthesis of target compounds is one of the important subjects in organic chemistry. Among them, biotransformations of exogenous substrates have been widely studied to synthesize chiral compounds [3–8]. Furthermore, the biotransformations help to lessen the environmental impact of organic syntheses. Microbial reduction of carbonyl compounds is a convenient method of obtaining optically pure alcohols. In particular, because of their bifunctional properties, hydroxy esters that are obtained from the reduction of keto esters are useful building blocks in organic synthesis and are employed as synthetic starting molecules for other chiral compounds [9–12]. For example, bakers' yeast (*Saccharomyces cerevisiae*) has often been used for the reduction of keto esters to obtain optically active hydroxy esters [13–18]. Furthermore, several keto ester reductases have been isolated from bakers' yeast and their enzymatic properties studied including the specific activities, the stereoselectivities, and kinetic parameters toward various keto esters [19–21]. Other microorganisms such as *Thermoanaerobactor brockii* (anaerobic bacterium) [22–24], *Geotrichum candidum* (fungi) [25–27], *Candida magnoliae* (yeast) [28] that can catalyze the stereoselective reduction of keto esters are also used for the preparation of chiral hydroxy esters. However, little information is known about the reduction of keto esters using other microbes (except for yeast and fungi).

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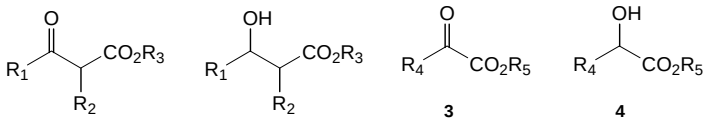
doi:10.1016/S1381-1177(03)00081-X



1 **2** **3** **4**

	R_1	R_2	R_3
a	$ClCH_2$-	H	Et
b	Me	H	Me
c	Me	H	Et
d	Me	Me	Et

	R_4	R_5
a	Me	Et
b	Et	Et
c	*n*-Pr	Et
d	*n*-Bu	Et
e	*n*-Pen	Et
f	*i*-Pr	Et
g	Ph	Me
h	Ph	Et

Scheme 1.

In this review, we introduce the possibility of microorganisms, in particular, aerobic thermophilic bacteria (*Bacillus* strains, thermophilic actinomycetes) and microalgae (*Chlorella* strains, marine algae) as new biocatalysts for the stereoselective reduction of α- and β-keto esters (Scheme 1). Furthermore, the mechanistic interpretation of the reduction is also discussed at the enzyme molecular levels.

2. Thermophilic *Bacillus* strains as biocatalysts

2.1. Screening of Bacillus strains

Selected thermophilic *Bacillus* strains were tested for the reducing activity of α- and β-keto esters [29]. The results of the conversion of β-keto esters (**1a**–**1d**) and α-keto esters (**3a**, **3f**, and **3h**) to the corresponding alcohols with ten *Bacillus* strains are summarized in Table 1. In the conventional method (without additive), the substrates (**1a**–**1d**, **3a**, **3f**, and **3h**) were reduced to the corresponding alcohol (**2a**–**2d**, **4a**, **4f**, and **4h**) with a low conversion ratio (as shown in parentheses in Table 1), while these ratios were increased by the introduction of glycerol in the reaction mixture as an additive. The addition of glycerol increased only the ratio in most cases tested; however, the introduction of other additives (citric acid, malic acid, glucose, 2,3-butanediol, 2-propanol, methanol, ethanol and 1-butanol) did not increase the ratio in the reduction.

Table 1
The conversions of substrates (**1a**–**1d**, **3a**, **3f**, and **3h**) to corresponding alcohols (**2a**–**2d**, **4a**, **4f**, and **4h**) by *Bacillus* strains[a,b]

Bacillus strains	DSM no.	β-Keto esters				α-Keto esters		
		2a	**2b**	**2c**	**2d**	**4a**	**4f**	**4h**
B. stearothermophilus	297	23 (20)[c]	8 (8)	29 (13)	73 (25)	55 (47)	99 (71)	89 (5)
B. stearothermophilus	457	34 (6)	32 (8)	7 (13)	32 (8)	64 (57)	34 (85)	75 (34)
B. smithii	460	22 (0)	13 (1)	11 (1)	1 (2)	8 (0)	99 (23)	85 (37)
B. sphaericus	461	9 (0)	14 (9)	8 (28)	78 (6)	89 (64)	93 (74)	69 (40)
Bacillus sp.	465	19 (13)	21 (5)	9 (6)	2 (2)	99 (79)	60 (36)	87 (28)
Bacillus sp.	466	42 (11)	19 (2)	39 (2)	6 (15)	70 (41)	71 (65)	77 (16)
B. stearothermophilus	494	13 (7)	5 (8)	5 (14)	50 (5)	91 (66)	67 (63)	74 (35)
B. thermocatenulatus	730	38 (11)	20 (6)	55 (3)	67 (4)	60 (37)	48 (31)	85 (60)
B. stearothermophilus	1550	7 (0)	17 (4)	80 (14)	57 (4)	41 (20)	98 (68)	90 (58)
B. stearothermophilus	2027	14 (4)	11 (6)	40 (39)	34 (6)	38 (29)	42 (29)	71 (42)

[a] The conversion ratios of the substrates to the corresponding alcohols were measured by GLC with a capillary column.
[b] The substrates (5 mM), glycerol (250 mM) and saline (20 ml) were added to the wet cells (0.2 g) and the reaction mixture were incubated at 37 °C for 20 h.
[c] The conversion ratios without glycerol are shown in parentheses.

2.2. Stereoselectivity

In the stereochemistry of products, the reduction of ethyl 2-methyl-3-oxobutanoate (**1d**) by three *Bacillus* strains (DSM 297, DSM 730, and DSM 2027) gave the corresponding β-hydroxy ester (**2d**) in high diastereoselectivity (*syn*/*anti* = 80–89/20–11), compared with a bakers' yeast reduction of the same substrate [21]. Further, the enantioselectivity of *syn*-(2*R*,3*S*)- and *anti*-(2*S*,3*S*)-**2d** reduced by *Bacillus* strains were >99 and >98% e.e., respectively. The ratio (80:20) of *syn*/*anti* of the product (**2d**) in the reduction by *Bacillus stearothermophilus* DSM 297 could be shifted to the extent of 98:2 by addition of $MgCl_2$, but the chemical yield was decreased to 12%. The ratio was not changed by introduction of other additives such as allyl alcohol, ethyl chloroacetate, or methyl vinyl ketone. Further, the reduction of ethyl 3-methyl-2-oxobutanoate (**3f**) by four *Bacillus* strains (DSM 297, DSM 461, DSM 466, and DSM 1550) also gave the corresponding α-hydroxy ester (**4f**) in high enantioselectivity as shown in Table 2. In particular, the reduction of **3f** with *B. stearothermophilus* DSM 297 afforded (*R*)-**4f** in high chemical yield (82%) with excellent stereoselectivity (>99% e.e.).

The mechanism for improvement of the conversion ratio by the addition of glycerol is not clear. It seems that the increase of the reduced nicotinamide-adenine dinucleotide (NADH or NADPH) due to the oxidative degradation of glycerol in the cells of these aerobic thermopiles would accelerate the stereoselective reduction of α- and β-keto esters to the corresponding optically pure alcohols.

3. Thermophilic actinomycetes as biocatalysts

3.1. Screening of actinomycetes

Several selected actinomycetes are tested for the reducing activity of α-keto esters [30]. The substrates (**3f** and **3g**) were converted to the corresponding alcohols (**4f** and **4g**) with nine strains. These results are summarized in Table 3. In the conventional method (without additive), the substrates were reduced to the corresponding alcohol with a low conversion ratio (as shown in parentheses in Table 3), while these ratios were increased by introduction of glycerol in the reaction mixture. Among the additives tested, glycerol only one effective compound to increase the ratio and other additives (citric acid, lactic acid, fumaric acid, malic acid, glucose, lactose, fructose, galactose, starch, alanine, glycine) did not increase the ratio.

3.2. Stereoselectivity

In the stereochemistry of products, the reduction of ethyl 3-methyl-2-oxobutanoate (**3f**) by three actinomycetes strains (NBRC 12133, NBRC 14178, and NBRC 14271) gave the corresponding (*R*)-ethyl 3-methyl-2-hydroxybutanoate (**4f**) with high enantioselectivity (>99% e.e.). On the other hand, the

Table 2
The reductions of β-keto esters (**1d**) and α-keto esters (**3f**) by *Bacillus* strains[a]

Ethyl 2-methyl-3-oxobutanoate (**1d**)						Ethyl 3-methyl-2-oxobutanoate (**3f**)			
Bacillus strains	DSM no.	Yield[b] (%)	*syn*/*anti*[c]	e.e.[d] (%)		*Bacillus* strains	DSM no.	Yield[b] (%)	e.e.[d] (%)
				syn-(2*R*,3*S*)	*anti*-(2*S*,3*S*)				
B. stearothermophilus	297	56	80/20	>99	99	*B. stearothermophilus*	297	82	>99 (*R*)
B. sphaericus	461	65	74/26	>99	96	*B. smithii*	460	85	7 (*S*)
B. thermocatenulatus	730	51	83/17	99	98	*B. sphaericus*	461	71	75 (*R*)
B. stearothermophilus	1550	36	51/49	>99	90	*Bacillus* sp.	466	60	79 (*R*)
B. stearothermophilus	2027	12	89/11	98	98	*B. stearothermophilus*	1550	79	72 (*R*)

[a] Saline (20 ml), the substrates (5 mM) and glycerol (250 mM) were added to the wet cells (0.2 g) and the reaction mixtures were incubated at 37 °C for 20 h.
[b] Isolated yields.
[c] The ratios of *syn*/*anti* were measured by GLC with a capillary column HR-20 M (0.25 mm × 25 m).
[d] The e.e. (%) and configuration were measured with GLC with an optically active capillary column Chiraldex G-TA (0.25 mm × 20 m).

Table 3
The conversion of substrates to the corresponding alcohols by actinomycetes whole cell[a]

Strains	NBRC no.	**3f**			**3g**		
		Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
Pseudonocardia thermophila	12133	74 (12)[d]	>99	*R*	81 (10)[d]	98	*R*
Saccharomonospora yiridis	12207	51 (41)	90	*R*	77 (15)	84	*R*
Streptomyces thermovulgaris	12383	44 (7)	89	*R*	66 (7)	87	*R*
Streptomyces thermovulgaris	13088	48 (20)	94	*R*	68 (10)	79	*R*
Thermoactinomyces monosporus	13920	55 (26)	94	*R*	8 (5)	86	*R*
Thermoactinomyces monosporus	14050	47 (8)	92	*R*	45 (8)	77	*R*
Thermomomospora mesouviformis	14178	71 (31)	>99	*R*	52 (11)	93	*R*
Streptomyces thermoluteus	14269	36 (15)	91	*R*	41 (8)	71	*R*
Streptomyces thermocyaneoviolaceus	14271	62 (14)	>99	*R*	75 (13)	98	*R*

[a] The substrate (5 mM), glycerol (250 mM), and saline (20 ml) were added to the wet cells (0.2 g) and the reaction mixture were incubated at for 20 h.
[b] The conversion ratios were measured by GLC analysis.
[c] The e.e. (%) and configuration were measured by GLC with an optically active capillary column.
[d] The conversion ratios without glycerol as an additive are shown in parentheses.

reduction of methyl benzoylformate (**3g**) was also catalyzed by two strains (NBRC 12133 and NBRC 14178) to methyl (*R*)-mandelate (**4g**) in high enantioselectivity (98% e.e.).

3.3. Immobilized cells

Repetitive use of whole cells as a biocatalyst was also investigated with Ca^{2+}-alginate immobilized *Pseudonocardia thermophila* NBRC 12133 (IMPT) as shown in Table 4 [30]. The used IMPT cells was recovered by filtration and washed with saline. The recovered IMPT cells were used as a biocatalyst of the next batch reaction. In the reduction using glycerol as an additive, the substrates (**3f** and **3g**) were reduced to the corresponding α-hydroxy esters with a low conversion ratio, in particular, the ratio of **3g** is less than 30% in every batch reaction.

When we also investigated the IMPT reduction of the substrates with other additives, the use of DL-alanine was better suited for the IMPT reduction than the use of glycerol as an additive. The conversion ratio of the IMPT reduction with DL-alanine did not decrease during the reuse of the immobilized cells.

Table 4
The conversion of substrates using immobilized actinomycetes with alanine[a]

Batch	**3f**				**3g**			
	Reaction time (h)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Reaction time (h)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
1	46	99	>99	*R*	20	93	98	*R*
2	42	99	>99	*R*	23	95	98	*R*
3	45	99	>99	*R*	20	96	99	*R*
4	30	98	>99	*R*	22	97	99	*R*
5	38	99	>99	*R*	21	98	98	*R*
6	44	98	>99	*R*	22	97	99	*R*
7	41	99	>99	*R*	20	94	98	*R*

[a] The substrate (5 mM), DL-alanine (250 mM), and saline (20 ml) were added to the immobilized cells (4 g) and the reaction mixture were incubated 37 °C.
[b] The conversion ratios were measured by GLC with a capillary column.
[c] The e.e. (%) and configuration were measured by GLC with an optically active capillary column.

Table 5
The reduction of α-keto esters (**3**) to the corresponding alcohols (**4**) with *S. thermocyaneoviolaceus* NBRC 14271[a]

Products	37 °C				45 °C				55 °C			
	Conversion[b] (%)	Yield[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	Yield[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	Yield[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
4a	>99	42	68	*S*	>99	41	74	*S*	>99	34	96	*S*
4b	>99	45	35	*S*	>99	43	62	*S*	>99	35	88	*S*
4c	>99	51	37	*S*	>99	49	22	*S*	99	33	20	*S*
4d	>99	53	28	*S*	>99	49	18	*S*	98	31	15	*S*
4e	>99	43	41	*S*	>99	45	23	*S*	95	35	17	*S*
4f	>99	57	>99	*R*	>99	53	63	*R*	98	35	45	*R*
4g	>99	68	98	*R*	>99	50	62	*R*	98	46	53	*R*
4h	>99	65	>99	*R*	>99	52	52	*R*	97	41	44	*R*

[a] Substrate (7.5 mM), glycerol (250 mM), and saline (20 ml) were added to the wet cell (0.5 g) and the reaction mixture was incubated for 20 h.
[b] Conversion and chemical yields were measured by GLC analysis.
[c] Enantiomeric excesses and configuration were measured by GLC analysis with optically active capillary columns.

Table 6
Effect of substrate concentration on the stereoselectivity of the reduction with *S. thermocyaneoviolaceus* NBRC 14271[a,b]

Products	Concentration of substrates (**3a**, **3f**, and **3h**)									
	2.5 mM		5.0 mM		7.5 mM		10 mM		15 mM	
	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
4a	86	*S*	79	*S*	74	*S*	54	*S*	47	*S*
4f	73	*R*	68	*R*	63	*R*	61	*R*	54	*R*
4h	69	*R*	65	*R*	62	*R*	45	*R*	41	*R*

[a] Substrate, glycerol (250 mM), and saline (20 ml) were added to the wet cell (0.5 g) and the reaction mixture was incubated for 20 h at 45 °C.
[b] Conversions were >99% in all cases (by GLC analysis).
[c] Enantiomeric excesses and configuration were measured by GLC analysis with optically active capillary columns.

In the IMPT reduction, the conversion ratio remained at a high value (>98%) for seven batch reductions. Further, (*R*)-**4f** and (*R*)-**4g** were produced with high enantioselectivity in every batch reaction (Table 4).

3.4. Effect of temperature on stereoselectivity

The reduction of the α-keto esters (**3a**–**3h**) with *Streptomyces thermocyaneoviolaceus* NBRC 14271 at various temperatures was investigated [31]. As shown in Table 5, ethyl 3-methyl-2-oxobutanoate (**3f**), methyl benzoylformate (**3g**), and ethyl benzoylformate (**3h**) were reduced to the corresponding α-hydroxy esters with high enantioselectivity (>99, 98, and >99% e.e., respectively) at 37 °C. The stereochemistry of the produced alcohols tended to change toward the (*S*)-configuration at high temperature. Interestingly, ethyl pyruvate (**3a**) and ethyl 2-oxobutanoate (**3b**) were reduced to the corresponding (*S*)-alcohols (**4a** and **4b**) with high enantioselectivity (96 and 88% e.e., respectively) at 55 °C, compared with the reduction at 37 °C (68 and 35% e.e., respectively). Such a change in the stereoselectivity of the produced alcohols at various temperatures has been reported [16,32]. The reduction of **3c**–**3e** at high temperature (55 °C) gave the corresponding alcohols (**4c**–**4e**) in low yield (17–20%).

3.5. Effect of substrate concentration on stereoselectivity

The effects of the substrate concentration on the reduction of three α-keto esters (**3a**, **3f**, and **3h**) with the actinomycete are summarized in Table 6 [31].

For the reduction of **3a**, when the concentration was low, the substrate was reduced to the corresponding alcohol with high 86% e.e. (*S*), however, the enantioselectivity decreased to a low e.e. (47% e.e.) with increasing concentration. On the other hand, **3f** and **3h** were reduced to the corresponding alcohols that had the (*S*)-configuration with increasing concentration.

3.6. Effect of additives on stereoselectivity

Table 7 shows the effect of various additives (mainly amino acids) on the reduction of **3** with *S. thermocyaneoviolaceus* [31]. The stereoselectivity of **4b** and **4c** were increased by the addition of alanine, asparagine, glutamic acid, aspartic acid, and malic acid. In particular, the reduction in the presence of asparagine and aspartic acid gave the corresponding (*S*)-alcohol in excellent enantioselectivity (>99% e.e.). It is noted that **3f**, **3g**, and **3h** were reduced to the (*R*)-alcohols with the actinomycete cells in the absence of additives, while the reduction in the presence of glutamic acid gave the antipodal (*S*)-alcohols in >99% e.e. Furthermore, the reduction of **3d** in the presence of aspartic acid gave the (*R*)-α-hydroxy ester (**4d**) in high e.e.

4. *Chlorella* strains as biocatalysts

4.1. Screening of Chlorella strains

Seven selected *Chlorella* strains were examined for their α- and β-keto ester reducing activities [33]. All of the *Chlorella* strains tested had the ability to

Table 7
Effect of additives on the stereoselectivity of produced α-hydroxy esters[a,b]

Products	Additives													
	Alanine		Glycine		Asparagine		Glutamic acid		Aspartic acid		Lactic acid		Malic acid	
	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
4a	84	*S*	83	*S*	90	*S*	89	*S*	90	*S*	72	*S*	85	*S*
4b	94	*S*	88	*S*	>99	*S*	82	*S*	>99	*S*	84	*S*	98	*S*
4c	>99	*S*	75	*S*	>99	*S*	>99	*S*	>99	*S*	46	*S*	>99	*S*
4d	7	*S*	30	*S*	28	*S*	27	*S*	86	*R*	71	*S*	43	*R*
4e	10	*S*	55	*S*	12	*S*	16	*R*	5	*S*	10	*S*	30	*R*
4f	35	*R*	40	*R*	15	*R*	>99	*S*	36	*R*	41	*R*	20	*R*
4g	82	*R*	79	*R*	86	*S*	>99	*S*	20	*R*	10	*R*	17	*R*
4h	60	*R*	58	*R*	>99	*S*	>99	*S*	35	*R*	30	*R*	26	*R*

[a] Substrate (7.5 mM), additive (500 mM, only asparagine: 100 mM), and saline (20 ml) were added to the wet cell (0.5 g) and the reaction mixture was incubated for 30 h at 37 °C.

[b] The conversions were >99% in all cases (by GLC analysis).

[c] Enantiomeric excesses and configuration were measured by GLC analysis with optically active capillary columns.

Table 8
Reduction of ethyl 2-methyl-3-oxobutanoate (**1**) by seven *Chlorella* strains[a]

Strains	IAM or SAG no.	Photoautotrophic cultivation				Heterotrophic cultivation			
		Conversion[b] (%)	*syn*/*anti*[b]	e.e.[c] (%)		Conversion[b] (%)	*syn*/*anti*[b]	e.e.[c] (%)	
				syn-(2*R*,3*S*)	*anti*-(2*S*,3*S*)			*syn*-(2*R*,3*S*)	*anti*-(2*S*,3*S*)
C. vulgaris	IAM C-27	41	64/36	59	20	39	60/40	70	19
C. fusca	IAM C-30	55	72/28	88	75	66	71/29	89	81
C. vulgaris	IAM C-135	38	73/27	68	79	49	69/31	82	66
C. saccharophila	IAM C-169	54	28/72	72	69	67	36/64	84	78
C. protothecoides	IAM C-206	57	16/84	90	92	65	7/93	98	96
C. vulgaris	IAM C-207	69	60/40	83	70	74	63/37	78	71
C. sorokiniana	SAG 211-8k	98	>99/<1	>99	–[d]	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] The ratio of *syn*/*anti* and the conversion were measured by GLC with a capillary column.
[c] The e.e. (%) and configuration were measured by GLC with an optically active capillary column.
[d] Not determined (not detected of the peak from the *anti*-hydroxy ester in GC analysis).

reduce ethyl 2-methyl-3-oxobutanoate (**1d**) and the α-keto esters (**3a**–**3f**, **3h**) as shown in Table 8. The algal reduction of **1d** with the heterotrophically cultivated *Chlorella* strains tended to slightly increase the conversion. The reduction **1d** by *Chlorella saccharophila* (IAM C-169) and *Chlorella protothecoides* (IAM C-206) 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 heterotrophically cultivated *C. protothecoides*. In the *syn*/*anti* ratio, the reduction by *Chlorella vulgaris* (IAM C-27, IAM C-135, IAM C-207), *Chlorella fusca* (IAM C-30), and *Chlorella sorokiniana* (SAG 211-8k) preferentially gave the corresponding *syn*-hydroxy ester. In particular, the reduction by the photoautotrophically cultivated *Chlorella sorokiniana* afforded the *syn*-hydroxy ester with excellent diastereo- (*syn* > 99%) and enantioselectivity (>99% e.e.).

The reduction of α-keto esters by *C. sorokiniana* that showed the best result in the reduction of **1d** was investigated (see Table 9). The reduction of **3** with the *C. sorokiniana* heterotrophically cultivated also tended to increase the conversion, however, the

Table 9
Reduction of α-keto esters by *Chlorella sorokiniana*[a]

Products	Photoautotrophic cultivation[b]			Heterotrophic cultivation[c]		
	Conversion[d] (%)	e.e.[e] (%)	Configuration[e] (*R*/*S*)	Conversion[d] (%)	e.e.[e] (%)	Configuration[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*
4h	98	>99	*R*	>99	23	*R*

[a] Saline (20 ml) and α-keto ester (**3a**–**3f**, **3h**) (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 non-glucose medium under light (1000 lx).
[c] *Chlorella* cells were grown in glucose-rich medium under light (1000 lx).
[d] The conversion was measured by GLC with a capillary column.
[e] The e.e. (%) and configuration were measured by GLC with an optically active capillary column.

Table 10
Effects of additives on the reduction of α-keto esters[a,b]

Additives	**4a**		**4f**		**4h**	
	Conversion[c] (%)	e.e.[d] (%)	Conversion[c] (%)	e.e.[d] (%)	Conversion[c] (%)	e.e.[d] (%)
No additive	>99	59 (*S*)	>99	17 (*S*)	>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**, **3f**, and **3h**) (7.5 mM), additive (75 mM) and 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 glucose-rich medium 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.

enantioselectivity of the products was low (below 68% e.e.). For the reduction of **3a** and **3h**, the photoautotrophic cultivated *C. sorokiniana* afforded the corresponding α-hydroxy ester (*S*)-**4a** and (*R*)-**4h** in >99% e.e., respectively.

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 photoautotrophically cultivated algal cells in the comparison with the those heterotrophically cultivated.

4.2. 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 [14]. For the reduction of three α-keto esters (**3a**, **3f**, and **3h**) by *C. sorokiniana*, the effects of additives on the conversion and enantioselectivity of the produced α-hydroxy esters were investigated (see Table 10) [33]. The conversion of the reduction from **3a** to **4a** in the presence of glycerol, glucose, and amino acids showed a high ratio (>99%), however, the enantioselectivity of the product **4a** was a low value (43–56% e.e.). The reduction of **3f** with glucose gave the corresponding (*R*)-hydroxy ester (**4f**) with 91% e.e. On the other hand, the reduction with ethyl propionate gave the (*S*)-hydroxy ester 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 increased the conversion and enantioselectivity of the product in the case of the reduction of ethyl benzoylformate (**4h**).

Ethyl 2-methyl-3-oxobutanoate (**1d**) was reduced by *C. sorokiniana* with glucose and amino acid to the corresponding β-hydroxy ester with a high conversion ratio, however, the diastereoselectivity was a low value. The algal reduction of **1d** 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 11. 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 an inhibitor for the enzyme(s) which produced one (*R*- or *S*-) enantiomer [19]. The increase of the reduced coenzyme (maybe NAD(P)H) by the oxidative degradation of the nutritious 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 [29,30].

Table 11
Effects of additives on the reduction of β-keto ester[a,b]

Additives	Conversion[c] (%)	*syn*/*anti*[c]	e.e.[d] (%)	
			syn-(2*R*,3*S*)	*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), additive (75 mM), and the wet cells (0.5 g) were incubated at 30 °C for 72 h under light (1000 lx).
[b] *Chlorella* cells were grown in glucose-rich medium 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.

5. Marine algae as biocatalysts

5.1. Screening of marine algae

Four selected marine algae were tested for reducing abilities toward α- and β-keto esters [34]. The results of the reduction of a β-keto ester (**1d**) and α-keto esters (**3a**–**3f**, 3**h**) are summarized in Tables 12 and 13, respectively. It was found that seven α-keto esters were converted to the corresponding α-hydroxy esters by the four marine algae. All marine algae reduced ethyl benzoylformate (**3h**) to the corresponding alcohol (**4h**) with a high conversion ratio, however, the enantioselectivity of the product **4h** showed a low enantiomeric excess. The reduction of ethyl 2-oxoheptanoate (**3e**) by *Chaetoceros gracilis* (EPFES-YU-1) gave the corresponding alcohol **4e** in high e.e. Ethyl 3-methyl-2-oxobutanoate (**3f**) was reduced by *Nannochloropsis* sp. (EPFES-YU-3) to (*R*)-**4f** in high e.e. (98%) with a high conversion ratio (99%).

The reduction of ethyl 2-methyl-3-oxobutanoate (**1d**) by the microalgae gave the corresponding *anti*-hydroxy ester (*anti*-**2d**) in low conversion ratios (25–68%). In particular, the substrate was reduced by *Nannochloropsis* sp. to the *anti*-hydroxy ester with excellent diastereo- (*syn*:*anti* = 1:99) and high enantioselectivity (>99%), when compared with the selectivity of **2d** reduced by *S. cerevisiae* [14], *Chlorella* [33], and *Glycine max* [35], which produced the *syn*-hydroxy ester predominantly.

5.2. Effects of additives

Furthermore, the effects of additives on the conversion ratio and the stereochemistry of the product (**4f**) were investigated as shown in Table 14 [34]. The reduction of **3f** by *C. gracilis* in the presence of glucose gave the corresponding α-hydroxy ester **4f** in a high conversion ratio (>99%); however, the enantioselectivity of the product was low (51%, *R*). In the algal reduction, the addition of DL-lactic acid decreased the conversion ratio, while the enantioselectivity of the product increased. The enantioselectivity of the product which was reduced under the acidic conditions (pH 3 and 5) was not changed (data not shown). In the presence of D-lactate ion, the substrate was not reduced. These results suggest that the D-lactate ion is an inhibitor for both the (*R*)- and (*S*)-α-hydroxy ester-producing enzyme(s), while the L-lactate ion inhibits only the activity of the (*S*)-enzyme(s).

6. Keto ester reductases from actinomycete

6.1. Purification of keto ester reductases

Ethyl 3-methyl-2-oxobutanoate was reduced by *S. thermocyaneoviolaceus* NBRC 14271 to the corresponding (*R*)-hydroxy ester with high e.e. at 37 °C, while the reduction at 55 °C produced the corresponding (*S*)-hydroxy ester [31]. In order to elucidate, the

Table 12
The reduction of α-keto esters by marine microalgae[a]

Products	*Chaetoceros gracilis*			*Chaetoceros* sp.			*Nannochloropsis* sp.			*Pavlova lutheri*		
	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	Conversion[b] (%)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
4a	99	50	*S*	99	9	*R*	99	16	*S*	48	48	*S*
4b	78	10	*S*	44	8	*S*	99	3	*R*	36	13	*S*
4c	64	15	*S*	51	27	*S*	99	52	*S*	28	25	*R*
4d	82	80	*S*	72	82	*S*	70	25	*S*	59	7	*S*
4e	42	89	*S*	40	56	*S*	71	3	*R*	18	8	*R*
4f	66	18	*S*	66	8	*S*	99	98	*R*	53	50	*S*
4h	99	23	*S*	99	17	*R*	99	21	*R*	99	4	*R*

[a] The synthetic seawater (20 ml) and α-keto ester (**3a**–**3f**, **3h**) (7.5 mM) were added to the wet cells (0.3 g) and the reaction mixtures were incubated at 20 °C for 24 h under light (1000 lx).

[b] The conversion was measured by GLC with a capillary column.

[c] The e.e. (%) and configuration were determined by GLC with an optically active capillary column.

Table 13
The reduction of β-keto esters by marine microalgae[a]

Marine algae	Conversion[b] (%)	*syn*/*anti*[b]	e.e.[c] (%)	
			syn-(2*R*,3*S*)	*anti*-(2*S*,3*S*)
Chaetoceros gracilis	25	30/70	98	>99
Chaetoceros sp.	41	18/82	97	>99
Nannochloropsis sp.	68	1/99	98	>99
Pavlova lutheri	40	21/79	96	>99

[a] Synthetic seawater (20 ml) and β-keto ester (**1d**) (7.5 mM) were added to the wet cells (0.3 g) and the reaction mixtures were incubated at 20 °C for 48 h under light (1000 lx).
[b] The conversion and *syn*/*anti* ratio were measured by GLC with a capillary column.
[c] The e.e. and configuration were determined by GLC with an optically active capillary column.

reaction mechanism, α-keto ester-reducing enzymes (STEKR-I, STEKR-II, and STEKR-III) have been isolated from the cell-free extract of *S. thermocyaneoviolaceus* [36,37].

STKER-I was purified from the cell-free extract by chromatographic methods, which included hydrophobic interaction, anion-exchange, affinity, and gel filtration chromatography. The overall 1043-fold purification was achieved from the crude cell-free extract with an overall yield of 16.3% [36]. STKER-II and STKER-III were purified *via* five chromatographic steps from the cell-free extract to homogeneity with an 8.2 and 1.4% overall recovery, respectively. The molecular mass of the native STKER-I was estimated to be 64 kDa. The molecular mass of the subunit size of the STKER-I was also estimated to be 30 kDa. The molecular masses of the native STKER-II and STKER-III were estimated to be 60 and 70 kDa, respectively. The molecular masses of the subunit were also estimated to be 29 and 30 kDa, respectively. These results showed that three enzymes (STKERs) consisted of two identical subunits (homodimer structure).

6.2. Substrate specificity

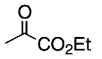
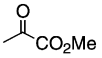
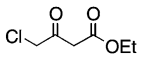
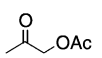
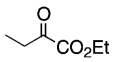
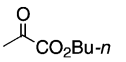
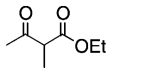
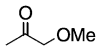
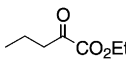
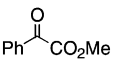
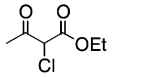
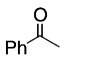
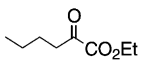
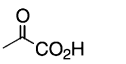
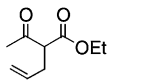
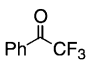
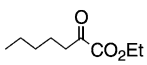
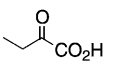
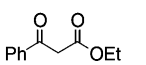
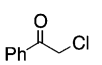
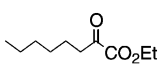
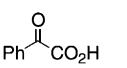
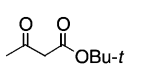
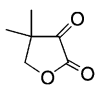
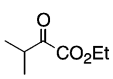
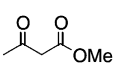
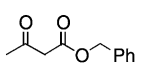
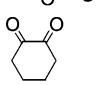
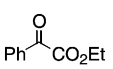
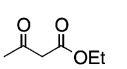
The substrate specificity of the STEKR-I toward various keto esters and ketones is summarized in Table 15. The enzyme catalyzed the reduction of various aliphatic α-keto esters having variable chain length and had low reducing activities toward aromatic α-keto esters (alkyl benzoylformate) and a few β-keto esters (such as ethyl 2-allylacetoacetate and ethyl 2-chloroacetoacetate). Furthermore, the enzyme did not catalyze the reduction of α-keto acid, acetophenone (and its derivatives), ketopantolactone, and cyclic diketone. These results show that the enzyme is specific for the reduction of α-keto esters. A carbonyl group at the α-position and an acyclic ester structure would be necessary for the high reducing activity of the enzyme.

Table 14
Effects of additives on the reduction of **3f**[a]

Additives	*Chaetoceros gracilis*		*Nannochloropsis* sp.	
	Conversion[b] (%)	e.e.[c] (%)	Conversion[b] (%)	e.e.[c] (%)
No additive	66	18 (*S*)	99	98 (*R*)
Glucose	>99	51 (*R*)	84	10 (*R*)
DL-Lactic acid	46	99 (*R*)	64	39 (*R*)
Lithium D-lactate	0	–	0	–
L-Lactic acid	89	99 (*R*)	60	49 (*R*)

[a] Synthetic seawater (20 ml) α-keto ester (7.5 mM), and 1.5 mmol of additive (75 mM) were added to the wet cells (0.5 g) and the reaction mixtures were incubated at 30 °C for 24 h under light (1000 lx).
[b] The conversion was measured by GLC with a capillary column.
[c] The e.e. and configuration were determined by GLC with an optically active capillary column.

Table 15
Substrate specificity of STKER-I from *S. thermocyaneoviolaceus* NBRC 14271

Substrates[a]	Relative rates[c] (%)	Substrate[a]	Relative rates[c] (%)	Substrate[b]	Relative rates[c] (%)	Substrate[b]	Relative rates[c] (%)
	100		98		2		0
	96		114		2		0
	50		12[b]		8		0
	61		0		7		0
	82		0		0		0
	86		0[b]		1		0
	9		1[b]		0		0
	2[b]		1[b]				

[a] Concentration was 1 mM.
[b] Concentration was 20 mM.
[c] Relative rates were determined by arbitrarily setting the activity reducing toward ethyl pyruvate to 100.

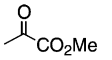
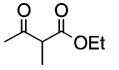
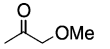
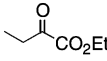
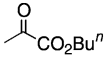
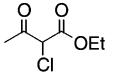
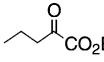
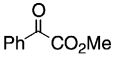
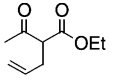
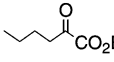
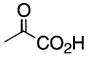
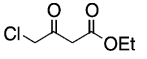
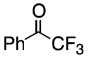
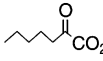
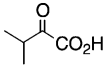
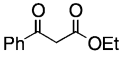
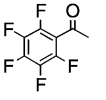
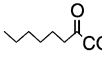
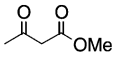
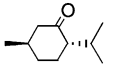
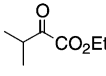
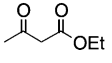
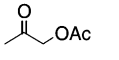
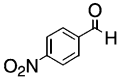
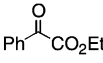
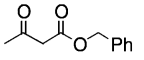
The relative activities of STKER-II and STKER-III toward α- and β-keto esters and other carbonyl compounds are also summarized in Table 16. STKER-II had greater activity toward aliphatic α-keto esters having a long alkyl chain such as **3d** and **3e**, while the activity for smaller substrates such as **3a** and methyl pyruvate was low. STKER-III showed high activity not only for aliphatic substrates but also for aromatic substrates. STKER-II had the reducing activity toward ethyl 4-chloroacetoacetate in β-keto esters we tested. STKER-III also had slight activities toward some β-keto esters (ethyl 2-chloroacetoacetate, ethyl 2-allylacetoacetate, ethyl 4-chloroacetoacetate, methyl acetoacetate, ethyl acetoacetate, ethyl 2-methyl acetoacetate, and ethyl benzoylacetate). The reducing activities by STKER-II and STKER-III for other substrates, such as benzyl acetoacetate, 1-acetoxy-2-propanone, 1-methoxy-2-propanone, acetophenone, propiophenone, 2′,3′,4′,5′, 6′-pentafluoroacetophenone, and (–)-menthone, were not observed. Both enzymes showed reducing activity toward neither α-keto acids such as pyruvic acid, 3-methyl butanoic acid, mandelic acid nor nitrobenzaldehyde, a typical substrate for aldo-keto reductases. These enzymes (STKER-II and STKER-III) were highly specific for NADPH as a sole coenzyme. Oxidation activity for ethyl lactate and ethyl 2-hydroxy-3-methyl butanoate was not found for both enzymes.

6.3. Stereospecificity

To clarify the stereochemistry of the products, the reduction of α-keto esters by STKER-I was carried out. As shown in Table 17, **3a**–**3f** and **3h** were reduced to the corresponding (*S*)-hydroxy esters with excellent e.e. (>99% e.e.). These results showed that both aliphatic and aromatic α-keto esters were reduced stereospecifically by STKER-I.

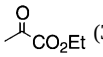
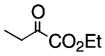
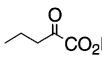
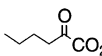
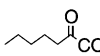
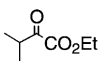
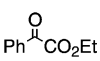
The reduction of α-keto esters by STKER-II and STKER-III was also carried out to clarify the stereochemistry of the products as shown in Table 17. The

Table 16
Substrate specificity of STKER-II and STKER-III from *S. thermocyaneoviolaceus* NBRC 14271[a]

Substrates	Relative rates[b] (%)		Substrate	Relative rates[b] (%)		Substrate	Relative rates[b] (%)		Substrate	Relative rates[b] (%)	
	STKER-II	STKER-III		STKER-II	STKER-III		STKER-II	STKER-III		STKER-II	STKER-III
	5	36		4	39		0	3		0	0
	20	60		25	71		– [c]	19		0	0
	42	100		7	73		0	7		0	0
	81	70		0	0		17	7		– [c]	29
	100	69		0	0		0	2		0	0
	53	59		0	3		8	– [c]		0	0
	59	34		0	4		0	0		0	0
	7	43		0	0						

[a] Concentration was 5 mM for α-keto esters and 20 mM for other substrates.
[b] Relative rates were calculated by setting the activity to be 100 (STKER-II; ethyl 2-oxoheptanoate, STKER-III; ethyl 2-oxopentanoate).
[c] Not measured.

Table 17
Stereoselectivity of STKER-I, STKER-II and STKER-III catalyzed reductions

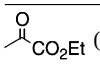
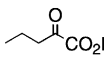
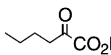
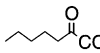
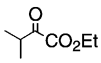
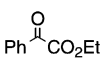
Substrates	STKER-I[a]		STKER-II[b]		STKER-III[b]	
	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)	e.e.[c] (%)	Configuration[c] (*R*/*S*)
 (**3a**)	>99	*S*	40	*R*	14	*S*
 (**3b**)	>99	*S*	86	*R*	38	*S*
 (**3c**)	>99	*S*	37	*R*	81	*S*
 (**3d**)	>99	*S*	34	*S*	71	*R*
 (**3e**)	>99	*S*	46	*S*	>99	*R*
 (**3f**)	>99	*S*	>99	*R*	>99	*R*
 (**3h**)	>99	*S*	31	*R*	>99	*R*

[a] The purified enzyme solution (10 units), NADPH (22 μmol), glucose 6-phosphate dehydrogenase (15 units), glucose 6-phophate (1.0 mmol), and the substrate (0.5 mmol) were incubated in 0.1 M potassium phosphate buffer (pH 7.0, 3 ml) for 24 h at 37 °C.

[b] The purified enzyme solution (1.0 unit), NADPH (10 μmol), and 0.1 M potassium phosphate buffer (pH 7.0, 0.5 ml) were incubated for 2 h at 37 °C.

[c] Enantiomeric excesses and configuration were measured by GLC analysis with optically active capillary columns.

Table 18
Kinetic parameters of STKER-I, STKER-II, and STKER-III from *S. thermocyaneoviolaceus* NBRC 14271

Substrates	STKER-I			STKER-II			STKWE-III		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
 (**3a**)	7.9×10^{-2}	5.7×10	7.2×10^{2}	3.2×10	5.3	1.7×10^{-1}	4.1	7.9×10	2.0×10
 (**3b**)	1.2×10^{-1}	9.5×10	7.8×10^{2}	2.2×10	5.7×10^{2}	2.6×10	1.5	1.2×10^{2}	7.9×10
 (**3c**)	1.6	5.3×10	3.4×10	4.5	4.9×10^{2}	1.1×10^{2}	2.9×10^{-1}	1.1×10^{2}	3.8×10^{2}
 (**3d**)	8.5×10^{-1}	5.2×10	6.1×10	2.5	7.4×10^{2}	3.0×10^{2}	9.5×10^{-1}	1.3×10^{2}	1.4×10^{2}
 (**3e**)	7.0×10^{-1}	7.2×10	1.0×10^{2}	8.3×10^{-1}	8.2×10^{2}	9.9×10^{2}	4.0×10^{-1}	1.3×10^{2}	3.2×10^{2}
 (**3f**)	9.0	4.2×10	4.6	3.0	5.5×10^{2}	1.9×10^{2}	8.1	1.6×10^{2}	1.9×10
 (**3h**)	1.6×10	7.8	5.0×10^{-1}	5.0	2.0×10^{2}	4.0×10	6.2×10^{-1}	1.1×10^{2}	1.7×10^{2}

enantioselectivity of the produced α-hydroxy esters by STKER-II was not as precise (from 46% e.e. (*S*) to 40% e.e. (*R*)) except for **3f** (>99% e.e. (*R*)) and **3b** (86% e.e. (*R*)). STKER-III reduced α-keto esters having a long alkyl chain, an isopropyl or a phenyl group to the corresponding (*R*)-hydroxy esters with excellent e.e. (such as **3e**, **3f**, **3g**, and **3h**), while the selectivity for **3a** and **3b** was low (14 and 38% e.e., respectively) and gave the (*S*)-hydroxy esters.

6.4. Kinetic constants

The kinetic parameters (K_m and k_{cat}) of STKER-I for aliphatic α-keto esters were listed in Table 18. The K_m values increased as the side alkyl chain in substrates was changed from methyl to *n*-propyl group (corresponding from **3a** to **3c**). However, the K_m value decreased with extension of the substituent from *n*-propyl to *n*-pentyl. These irregular changes in the K_m values reflect their relative activity (see Table 18). The K_m and k_{cat} values of **3f** were 9.0 mM and $8.3 \times 10\,s^{-1}$, respectively, which indicated that the enzyme had low affinity for the substrate. This would be due to the steric effects of the bulky isopropyl group (at the β-position site) in the substrate. The K_m values of NADPH and NADH were 2.3×10^{-2} and 5.3×10^{-1} mM, respectively. When the reduction of ethyl pyruvate by the purified enzyme occurred, the reaction rate with NADPH as a coenzyme was faster than that with NADH. Although the enzyme is able to use both coenzymes, NADPH would be utilized mainly as a coenzyme in the cell. The kinetic constants of STKER-II and STKER-III are summarized as shown in Table 18. The K_m values of STKER-II toward **3a**, **3b**, **3c**, **3d**, and **3e** were 3.2×10, 2.2×10, 4.5, 2.5, and 8.3×10^{-1} mM, respectively. As the alkyl chain in the substrate molecule became longer, the corresponding K_m value of STKER-II was decreased. On the contrary, the k_{cat} value tended to become larger with extension of the substituent. Thus, STKER-II prefers substrates having a long alkyl chain.

STKER-III showed remarkable reducing ability for aromatic α-keto esters. The k_{cat}/K_m value for ethyl benzoylformate was larger than those of STKER-I and STKER-II in the actinomycete cells. These results suggest that STKER-III contributes mainly to reduction of ethyl benzoylformate to give the corresponding (*R*)-α-hydroxy ester in actinomycete cells.

6.5. Stability

The thermostability of STKER-I, STKER-II, and STKER-III are shown in Fig. 1. The STKER-I showed

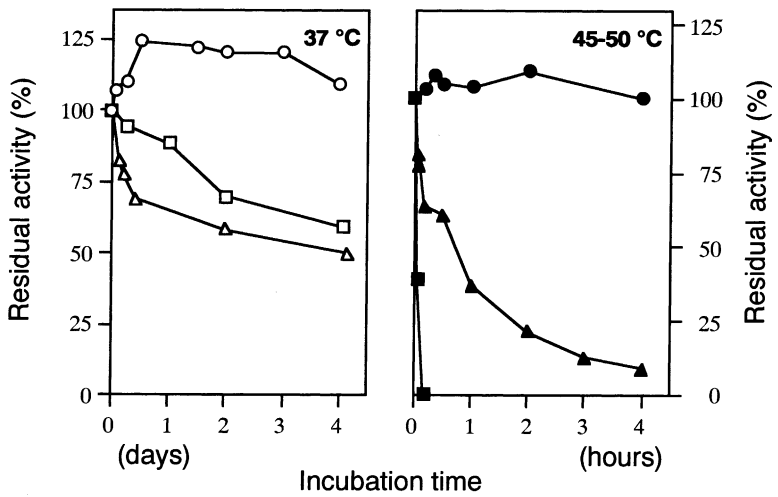
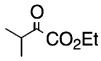


Fig. 1. The thermostability of STKERs from *S. thermocyaneoviolaceus*. The stability of the enzyme activity (residual activity) was measured in 0.1 M potassium phosphate buffer (pH 6.5) at 37 °C after incubation at 37, 45, and 50 °C. After the incubation, the enzyme activity was immediately assayed. The relative activities were the values expressed as percent to the activity without incubation. (○) STKER-I incubated at 37 °C, (△) STKER-II incubated at 37 °C, (□) STKER-III incubated at 37 °C, (●) STKER-I incubated at 50 °C, (▲) STKER-II incubated at 45 °C, (■) STKER-III incubated at 45 °C.

Table 19
Comparison of the characteristics of the reductases from various microorganisms

Enzyme (source)	Molecular mass						Coenzyme
	GFC[a] (kDa)	SDS-PAGE (kDa)	K_m (mM)	e.e. (*R*/*S*)	K_m (mM)	e.e. (*R*/*S*)	
STKER-I (*S. thermocyaneoviolaceus*)	64	30 (dimer)	7.9×10^{-2}	>99 (*S*)	9.0	>99 (*S*)	NADH/ NADPH
STKER-II (*S. thermocyaneoviolaceus*)	60	29 (dimer)	3.2×10	40 (*R*)	3.0	>99 (*R*)	NADPH
STKER-III (*S. thermocyaneoviolaceus*)	70	30 (dimer)	4.1	14 (*S*)	8.1	>99 (*R*)	NADPH
YKER-II (Bakers' yeast) [21]	58	29 (dimer)	1.4×10^{2}	98 (*S*)	N.R.[b]	N.R.[b]	NADPH
YKER-IV (Bakers' yeast) [21]	31	39 (monomer)	4.3×10^{-1}	>99 (*R*)	2.7×10^{-1}	>99 (*R*)	NADPH
YKER-V (Bakers' yeast) [21]	83	41 (dimer)	5.1	94 (*S*)	7.9×10	77 (*S*)	NADPH
Carbonyl reductase (*C. magnoliae*) [28]	76	32 (dimer)	2.1×10	–[c]	2.4×10^{2}	–[c]	NADPH

[a] GFC: gel filtration chromatography.
[b] N.R. indicates no reaction.
[c] Not determined.

high stability at 37 °C. After 4 days, only a 12% loss of activity was detected at 37 °C. Storage at 4 °C for 3 months caused no loss of activity. Furthermore, the enzyme retained above 60% of its initial activity after 12 h at 50 °C. From the viewpoint of stability, STKER-I will serve mainly in the reduction of α-keto esters with *S. thermocyaneoviolaceus* cells at high temperature (60 °C) [30]. STKER-II and STKER-III retained 50–60% activity after 4 days incubation at 37 °C. The stabilities of STKER-II, STKER-III were similar under incubation at 37 °C. After incubation at high temperature (STKER-II and STKER-III, 45 °C; STKER-I, 50 °C), STKER-II and STKER-III were deactivated for several hours.

6.6. Comparison with other microbial keto ester reductases

Several α-keto ester reducing enzymes have been isolated from microorganisms such as *S. cerevisiae* (bakers' yeast) [21] and *C. magnoliae* [28]. The properties of these enzymes are compared with those of the purified enzyme in this study as shown in Table 19. Although other enzymes including STKER-II and STKER-III utilized NADPH as a sole coenzyme, STKER-I utilized both NADPH and NADH as the coenzyme. STKER-I had high affinity for ethyl pyruvate among the listed reductases. Furthermore, STKER-I reduced **3f** to the corresponding

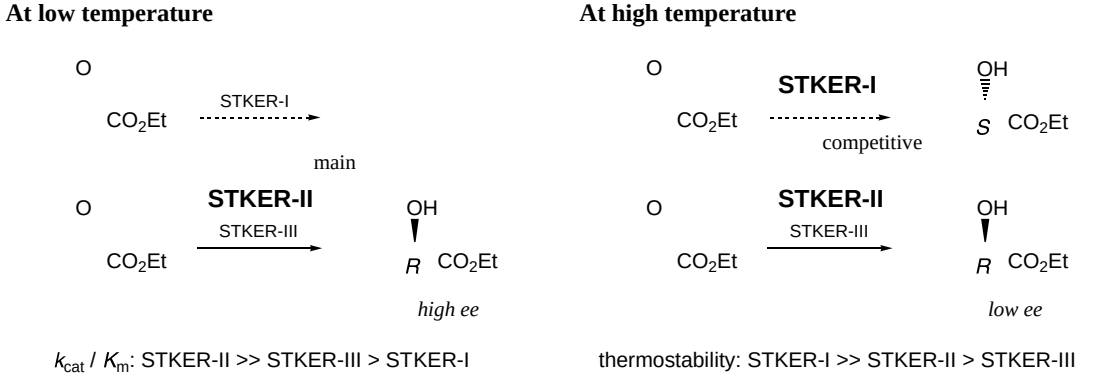


Fig. 2. The reduction of ethyl 3-methyl-2-oxobutanoate (**3f**) by *S. thermocyaneoviolaceus* whole cells.

(*S*)-hydroxy ester in excellent e.e. (>99% e.e.). Therefore, STKER-I would be very useful for catalysis of the preparation of ethyl (*S*)-**4f**. STKER-II and STKER-III reduced **3f** to the corresponding (*R*)-hydroxy ester with excellent e.e. as did YKER-IV from bakers' yeast.

6.7. Reaction mechanism

The reduction of **3f** by the whole cells at 37 °C gave the corresponding (*R*)-hydroxy esters. This result suggests that the substrate was reduced mainly by STKER-II (the lowest K_m value of the three reductases) in the actinomycete cells. However, the actinomycete reduction product at high temperature corresponded to the (*S*)-hydroxy ester. It is presumed that this stereoselectivity change may be attributed to differences in the thermostability of the reductases which contribute to the reduction in the cells, that is, because the thermostability of STKER-I is higher than that of STKER-II and STKER-III, STKER-I contributes mainly to the reduction at high temperature, consequently, the corresponding (*S*)-hydroxy ester was produced at 55 °C (as shown in Fig. 2). The stereoselectivity change in the reduction of ethyl pyruvate and ethyl benzoylformate would be explicable in the same manner mentioned above.

7. Conclusions

In this review, we reported that aerobic thermophilic bacteria (*Bacillus* strains and thermophilic actinomycetes) and microalgae (*Chlorella* strains and marine algae) had high reducing abilities toward keto esters and were available as a tool for stereoselective reduction of α- and β-keto esters as well as yeast and fungi.

Henceforth, novel and useful biocatalysts for organic syntheses will be discovered. Designed and desired enzymes will also be created by functional modification using genetic engineering methods. Furthermore, not only microorganisms but also plant cultured cells (or animal cells) will be utilized as biocatalysts. In the near future, new screening based on genetic information is expected to be one method for searching for useful biocatalysts.

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