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Biocatalytic Preparation of Chiral Hydroxy Esters using Entomogenous Fungi: Bio-reduction of Keto Esters by Tochukaso and Related Species

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A B S T R A C T

To research the potential ability of tochukaso and related species to act as biocatalysts, we screened 10 entomogenous fungal strains. The recommended medium (Potato Dextrose broth) and a synthetic medium (PGO medium) were tested as the liquid media for culturing these fungi. Four strains (NBRC33061, 100684, 103832, and 109003) cultured using the PGO medium showed a good growth. The stereoselective reduction of α- and *β*-keto esters by these four strains was also investigated, and they were found to be able to reduce various α-keto esters. Specifically, the reduction of α-keto esters by the PGO-cultivated *Isaria cicadae* NBRC33061 strain in the presence of L-glutamate as an additive yielded corresponding α-hydroxy esters with a high conversion ratio and in excellent enantioselectivity. Furthermore, it was found that the PGO-cultivated NBRC109003 strain in the presence of L-glutamate stereospecifically reduced ethyl 2-methyl-3-oxobutanoate to (2*S*, 3*S*)-2-methyl-3-hydroxybutanoate, i.e., only one of the four theoretically possible isomers. Overall, tochukaso and related species were shown to have a great potential for application as biocatalysts for the stereoselective reduction of carbonyl compounds.

Introduction

In a narrow sense, *Ophiocordyceps sinensis* (an entomopathogenic fungus formerly known as *Cordyceps sinensis*), which parasitizes the larvae of the flying fox moth distributed in the Tibetan Plateau in Tibet and the Himalayan regions of Bhutan and Nepal at 3,500 meters above sea level, is the only natural species generally called "tochukaso" (summer grass, winter worm) and has been used in traditional Chinese medicine (Mizuno, 1999; Coates *et al.*, 2005). On the other hand, in a broader sense, "tochukaso" refers to molds that parasitize the larvae of cicadas and moths in general, and many types have been discovered in Japan. The medicinal and pharmacological applications of *Cordyceps* mushrooms have been reported, while the possibility of its

application of these species in other fields related to entomogenous fungi has not been sufficiently explored. To discover novel biocatalysts, we have previously studied the microbial production of useful substances such as optically active compounds and clarified the substance conversion abilities (especially, the asymmetric reduction of carbonyl compounds) of yeasts, fungi, green algae, and bacteria (actinomycetes) (Ishihara *et al.,* 1995; Ishihara *et al.,* 2003; Ishihara *et al.,* 2006; Ishihara *et al.,* 2010; Ishihara *et al.,* 2011a; Ishihara *et al.,* 2011b; Ishihara *et al.,* 2012).

Previously, we also investigated the potential activity of tochukaso and related species (13 entomogenous fungal strains belonging primarily to genus *Elaphocordyceps*) as biocatalysts for the asymmetric reduction of carbonyl compounds, and found that several strains had an excellent reduction ability (Ishihara *et al.,* 2013). However, the potential biocatalyst activity of other tochukaso and their related species (such as *Ophiocordyceps*, *Isaria*, and *Metarhzium* spp.) has not been investigated.

This study describes the stereoselective reduction of α- and β-keto esters by tochukaso and related species (Clavicipitaceae) acting as novel biocatalysts (Figures 1 and 2).

Materials and Methods

Instruments and Chemicals

Gas chromatography (GC) was performed using a GL Science GC-353 gas chromatograph (GL Science Inc., Tokyo, Japan) equipped with capillary columns (DB-Wax, Agilent Technologies, Santa Clara, CA, USA, 0.25 μm, 0.25 mm x 30 m; TC-1, GL Science Inc., 0.25 μm, 0.25 mm x 30 m; CP-Chirasil-DEX CB, Varian Inc., Lake Forest, CA, USA, 0.25 μm, 0.25 mm x 25 m; Gamma DEX 225, Sigma-Aldrich Co., St. Louise, MO, USA, 0.25 μm, 0.25 mm x 30 m). Ethyl pyruvate (Figure 1,**1a**), diatomaceous earth (granular), Daigo's potato dextrose agar (PDA), olive oil and polypepton were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Difco™ potato dextrose broth (PDB) and Bacto™ yeast extract were purchased from Becton, Dickinson and Co., Franklin Lakes, NJ, USA.

Ethyl lactate (**2a**), ethyl 3-methyl-2-oxobutyrate (**1f**), ethyl 2-oxo-4-phenylbutyrate (**1h**), ethyl 2-hydroxy-4-phenylbutyrate (**2h**), and arabic gum were purchased from Sigma-Aldrich. Ethyl benzoylformate (**1g**), ethyl mandelate (**2g**) and ethyl 2-methyl-3-oxobutanoate (Figure 2, **3**) were obtained from Tokyo Chemical Industry, Co. Ltd., Tokyo, Japan. Ethyl 2-oxobutanoate (**1b**), ethyl 2-oxopentanoate (**1c**), ethyl 2-oxohexanoate (**1d**), ethyl 2-oxoheptanoate (**1e**), and hydroxy esters (**2b-f**, **4**) were prepared according to the procedures described in the literature (Nakamura *et al.,* 1988).

Microorganisms and Culture

The entomogenous fungi *Isaria cicadae* NBRC33061 (Japanese name: tsukutsukuboshi-dake), *Isaria farinosa* NBRC100647 (Japanese name: konasanagi-take), *Metarhizium indigoticum* NBRC100684 (Japanese name: midoritosaka-take), *Cordyceps pluricaptata* NBRC100745 (Japanese name: usukitanposemi-take), *Cordyceps* cf. *hepialidicola* NBRC100943 (Japanese name: kusagimushi-take), *Ophiocordyceps annullata* NBRC101738 (Japanese name: himekuchikitanpo-take), *Cordyceps caldinalis* NBRC103832 (Japanese name: hosoenokobenimushi-take), *Cordyceps tuberculata* NBRC106948 (Japanese name: suzumega-take), *Ophiocordyceps sobolifera* NBRC106967 (Japanese name: semi-take), and *Cordyceps puruinosa* NBRC109003 (Japanese name: himesanagi-take) were purchased from the National Institute of Technology and Evaluation, Biological Resource Center (NBRC, Japan).

These strains were maintained at 25°C in PDA, while they were grown in a 200mL culture of PDB and a synthetic medium (PGO medium) for 10 days at 25°C with aerobic rotary shaking at 120 rpm in a baffled 500mL flask in the dark. The PGO medium contained 20 g of glucose, 4 g of polypepton, 1 g of Bacto™ yeast extract, 0.46 g of KH_2PO_4, 1 g of K_2HPO_4, 2 g of pectin, 2 g of arabic gum, and 10 g of olive oil per liter of distilled water (pH 5.5) (Ishihara *et al.,* 2013). The fungalwet cells were harvested by filtration on a filter paper (Whatman, No. 4) *in vacuo* and washed with saline solution (0.85% aqueous NaCl).

Reduction of α-and β-Keto Esters with Resting Cells of Entomogenous Fungi

The saline-washed wet cells (0.5 g, dry weight approximately 0.15 g) were resuspended in a large test tube (ϕ 30 mm x 200 mm) containing 20 mL of saline. The substrate (0.15 mmol; 7.5 mM) and additive (7.5 mmol) were added, and the reaction mixture was incubated aerobically at 25°C under reciprocating

shaking at 120 rpm. A portion (0.5 mL) of the mixture was filtered using a short diatomaceous earth column (ϕ 10 mm x 30 mm), extracted with diethyl ether (5.0 mL), and then concentrated under reduced pressure.

Analysis

The conversions of the produced alcohols (Figure 1, **2a-h**) were measured using a GLC with a DB-WAX capillary column (100 kPa He at 110°C:**1a**, 3.78 min; **2a**, 4.75 min; **1b**, 4.73 min; **2b**, 5.92 min; **1f**, 4.54 min; **2f**, 6.41 min; 120°C; **1c**, 4.84 min; and **2c**, 6.45 min;**3**, 5.54 min; **4**-*anti*, 7.62 min; **4**-*syn*, 8.13 min; 150°C:**1d**, 3.83 min; **2d**, 4.68 min; **1e**, 4.78 min; and **2e**, 6.07 min; 180°C: **1g**, 9.01 min; and **2g**, 12.08 min) or a TC-1 capillary column (100 kPa He at 140°C: **1h**, 10.02 min; **2h**, 10.96 min).

The enantiomeric excess (e.e.) of the product was measured using a GC instrument equipped with an optically active CP-Chirasil-DEX CB (**2a**-**e**, **2g**-**h**, and **4**) or a Gamma DEX 225 capillary column (**2f**). The following equation was used for calculations: e.e.(%) = {(*R*-*S*)/(*R*+*S*)} x 100, where *R* and *S* are the respective peak areas detected in the GC analyses. The absolute configurations of the α- and β-hydroxy esters (**2a**-**h** and **4**) were identified by comparing the retention times derived from the GC analyses with those of the authentic samples (Nakamura *et al.,* 1988).

Results and Discussion

Screening of Entomogenous Fungi and Culture Media

The suitable medium to be used for the liquid culture was determined by measuring the amounts of wet cells obtained by culturing the 10 entomogenous fungi in two culture media (as shown in Table 1). The recommended medium for most of the strains tested in this study was PDA medium. All 10 strains were aerobically cultured in liquid PDB medium (the recommended medium without agar). Among them, *I. cicadae* NBRC33061 produced over 2.5 g of wet cells per 200 mL of liquid culture medium; however, the other nine strains cultivated in the same medium yielded a quantity of 1.7 g or less of wet cells. In the previous study, reported that PGO medium (which is a synthetic medium) is suitable for the liquid cultivation of entomogenous fungi (Ishihara *et al.,* 2013) and basidiomycetes such as edible mushrooms (Ishihara *et al.,* 2012, 2019).

Culturing the 10 strains in the PGO medium resulted in a significant enrichment in the yield of wet cells in four strains (i.e., *I. cicadae* NBRC33061, *M. indigoticum* NBRC100684, *C. caldinalis* NBRC103832, and *C. puruinosa* NBRC109003), and in particular, the yield of NBRC33061 strain increased to 3.5 g per 200 mL of culture medium.

Therefore, we investigated the possibility of these four tochukaso and related species acting as biocatalysts for the asymmetric reduction of carbonyl compounds.

Reduction of α-Keto Esters by Tochukaso and Related Species

The four high-yielding strains (i.e., NBRC33061, 100684, 103832, and 109003) cultivated in two liquid media (PDB and PGO) were tested for their ability to reduce α-keto esters (Figure 1). The tests involved several α-keto esters, and the results are summarized in Tables 2 and 3. All four tochukaso and related species were shown to reduce α-keto esters (**1a**-**h**) to the corresponding α-hydroxy esters (**2a**-**h**). Among the four strains cultured in PDB medium, strain NBRC33061 was shown to reduce three substrates at a high conversion rate (>99%), whereas low conversion rates were observed for most of the substrates reduced by the other strains (Table 2). The four strains were shown to reduce substrates at higher conversion ratio compared when cultivated in the PGO medium than under cultivation in the PDB medium (Table 3). In particular, it was found that strain NBRC33061 reduced all substrates and five substrates at conversion rates of >80% and >99%, respectively (**1a**-**c**, **1f**, and **1g**). However, the stereoselectivity of the produced hydroxy esters remained at around 80% except for the produced alcohols having short alkyl chain (**2a** and **2b**).

In the microbial reduction of carbonyl compounds by common bakers' yeast or filamentous fungi, it is well known that the introduction of small organic molecules or metal ions will increase the stereoselectivity of the produced alcohols (Kawai *et al.,* 1994; Kawai *et al.,* 1995; Nakamura *et al.,* 1996). In contrast, in the reduction by actinomycetes, several studies have reported that the addition of amino acids or sugars is effective in improving the conversion rate and the stereoselectivity of the products (Ishihara *et al.,* 2000; Ishihara *et al.,* 2003; Ishihara *et al.,* 2010; Ishihara *et al.,* 2011b).

Table.1 The cultivation of entomogenous fungi in two culture media

Scientific name	NBRC No.	Wet cells (g / 200-mL of culture)	
		PDB medium[1]	PGO medium[2,3]
Isaria cicadae	33061	2.6	3.5
Isaria farinosa	100647	0.9	0.7
Metarhizium indigoticum	100684	1.1	2.1
Cordyceps pluricaptata	100745	<0.1	0.7
Cordyceps* cf. *hepialidicola	100943	0.9	1.8
Ophiocordyceps annullata	101738	<0.1	0.3
Cordyceps caldinalis	103832	1.7	2.9
Cordyceps tuberculata	106948	<0.1	0.4
Ophiocordyceps sobolifera	106967	<0.1	0.2
Cordyceps puruinosa	109003	1.6	2.5

[1]The entomogenous fungi were grown in the medium at 25°C for 10 days with aerobic rotary shaking (120 min^{-1}) in a baffled 500-mL flask in the dark condition.
[2]Composition of PGO medium was described in materials and method section.
[3]The entomogenous fungi were grown in the medium at 25°C for 10 days with aerobic rotary shaking (120 min^{-1}) in a baffled 500-mL flask in the dark condition.

Table.2 The reduction of α-keto esters (**1a**-**h**) to α-hydroxy esters (**2a**-**h**) with four entomogenous fungi cultivated in PDB medium[1].

Product	*Isaria cicadae* NBRC33061			*Metarhizium indigoticum* NBRC100684			*Cordyceps caldinalis* NBRC103832			*Cordyceps puruinosa* NBRC109003		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	7	27	*S*	6	26	*S*	4	34	*S*	18	14	*S*
2b	98	>99	*R*	96	>99	*R*	93	>99	*R*	64	54	*R*
2c	>99	78	*S*	87	42	*R*	97	1	*S*	79	35	*R*
2d	95	74	*S*	95	24	*R*	97	27	*S*	79	1	*S*
2e	<1	37	*S*	27	11	*S*	23	30	*S*	17	55	*S*
2f	>99	34	*S*	>99	80	*R*	>99	36	*R*	96	78	*R*
2g	>99	74	*S*	78	96	*R*	52	93	*R*	87	62	*R*
2h	60	71	*S*	74	47	*S*	32	74	*R*	35	>99	*S*

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically (reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion was measured by a GLC analysis.
[3]Enantiomeric excess (e.e.) and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Table.3 The reduction of α-keto esters (**1a**-**h**) to α-hydroxy esters (**2a**-**h**) with four entomogenous fungi cultivated in PGO medium[1].

Product	*Isaria cicadae* NBRC33061			*Metarhizium indigoticum* NBRC100684			*Cordyceps* cf. *hepialidicola* NBRC100943			*Cordyceps caldinalis* NBRC103832			*Cordyceps puruinosa* NBRC109003		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	>99	>99	*S*	98	76	*S*	96	64	*S*	98	78	*S*	89	64	*S*
2b	>99	>99	*S*	99	96	*S*	94	>99	*S*	79	84	*S*	82	79	*R*
2c	>99	88	*S*	92	18	*R*	90	34	*R*	92	60	*R*	74	22	*R*
2d	98	84	*S*	88	24	*R*	74	19	*R*	89	56	*R*	68	13	*S*
2e	80	79	*S*	70	76	*S*	63	26	*R*	76	38	*S*	42	39	*S*
2f	>99	81	*S*	>99	90	*S*	>99	85	*S*	97	82	*S*	94	86	*S*
2g	>99	86	*S*	83	68	*S*	64	38	*S*	86	88	*S*	88	70	*S*
2h	88	88	*S*	72	44	*S*	26	47	*S*	48	69	*S*	40	81	*S*

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g) cultured in PGO medium, and the reaction mixture was incubated aerobically(reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion was measured by a GLC analysis.
[3]Enantiomeric excess (e.e.) and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Table.4 Effects of the additives on the reduction of α-keto esters (**1a**-**h**) to α-hydroxy esters (**2a**-**h**) by *I. cicadae* NBRC33061[1].

Product	Cultivation in PDB medium						Cultivation in PGO medium					
	L-Alanine			Sodium hydrogen L-glutamate			L-Alanine			Sodium hydrogen L-glutamate		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	<1	52	*S*	94	>99	*S*	>99	>99	*S*	>99	>99	*S*
2b	94	77	*S*	96	98	*S*	>99	98	*S*	>99	>99	*S*
2c	79	55	*S*	94	88	*S*	>99	94	*S*	98	>99	*S*
2d	90	18	*S*	90	74	*S*	87	83	*S*	93	95	*S*
2e	36	45	*S*	82	66	*S*	80	59	*S*	95	94	*S*
2f	81	27	*S*	97	85	*S*	>99	>99	*S*	>99	>99	*S*
2g	83	29	*S*	88	72	*S*	79	93	*S*	>99	92	*S*
2h	56	>99	*R*	81	89	*S*	72	84	*S*	92	>99	*S*

[1]Substrate (0.15 mmol), 0.85% NaCl aq. (20 ml) and additive (5 mmol) were added to the wet cells (0.5 g) cultured in PDB or PGO medium, and the reaction mixture was incubated aerobically (reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion was measured by a GLC analysis.
[3]Enantiomeric excess (e.e.) and absolute configuration (*R*/*S*) were determined by GLC analyses with optically active capillary columns.

Table.5 The reduction of ethyl 2-methyl-3-oxobutanoate (**3**) to the corresponding *β*-hydroxy ester (**4**) with four entomogenous fungi cultivated in PDB medium[1].

Scientific name	NBRC No.	conv. (%)[2]	*Syn* / *Anti*[2]	enantiomeric excess (%)[3]	
				Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
Isaria cicadae	33061	91	14 / 86	>99	71
Metarhizium indigoticum	100684	<1	---[4]	---[4]	---[4]
Cordyceps caldinalis	103832	<1	---[4]	---[4]	---[4]
Cordyceps puruinosa	109003	93	8 / 92	>99	>99

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically(reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion and *syn*/*anti* ratio were measured by a GLC analysis.
[3]Enantiomeric excess and absolute configuration were determined by GLC analyses with optically active capillary columns.
[4]Not determined

Table.6 The reduction of ethyl 2-methyl-3-oxobutanoate (**3**) to the corresponding *β*-hydroxy ester (**4**) with four entomogenous fungi cultivated in PGO medium[1].

Scientific name	NBRC No.	conv. (%)[2]	*Syn* / *Anti*[2]	enantiomeric excess (%)[3]	
				Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
Isaria cicadae	33061	86	10 / 90	>99	81
Metarhizium indigoticum	100684	38	22 / 78	>99	87
Cordyceps caldinalis	103832	11	26 / 74	>99	64
Cordyceps puruinosa	109003	88	5 / 95	>99	93

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically(reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion and *syn*/*anti* ratio were measured by a GLC analysis.
[3]Enantiomeric excess and absolute configuration were determined by GLC analyses with optically active capillary columns.

Table.7 Effects of the additives on the reduction of ethyl 2-methyl-3-oxobutanoate (**3**) to the corresponding *β*-hydroxy ester (**4**) with *Cordyceps puruinosa* NBRC109003 cultivated in PDBand PGO media[1].

Additive	Cultivation in PDB medium				Cultivation in PGO medium			
	conv. (%)[2]	*Syn* / *Anti*[2]	enantiomeric excess (%)[3]		conv. (%)[2]	*Syn* / *Anti*[2]	enantiomeric excess (%)[3]	
			Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)			*Syn*-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
L-Alanine	84	11 / 89	>99	79	88	4 / 96	>99	88
Sodium hydrogen L-glutamate	92	6 / 94	>99	94	>99	<1 / >99	---[4]	>99

[1]Substrate (0.15 mmol), additive (5 mmol), 0.85% NaCl aq. (20 ml) were added to the wet cells (0.5 g) cultured in PDBor PGO medium, and the reaction mixture was incubated aerobically(reciprocating shaking at 120 min^{-1}) at 25 °C for 48 hrs.
[2]Conversion and *syn*/*anti* ratio were measured by a GLC analysis.
[3]Enantiomeric excess and absolute configuration were determined by GLC analyses with optically active capillary columns.
[4]Not determined.

Figure.1 The reduction of α-keto esters (**1a**-**h**) to corresponding α-hydroxy esters (**2a**-**h**) by entomogenous fungi

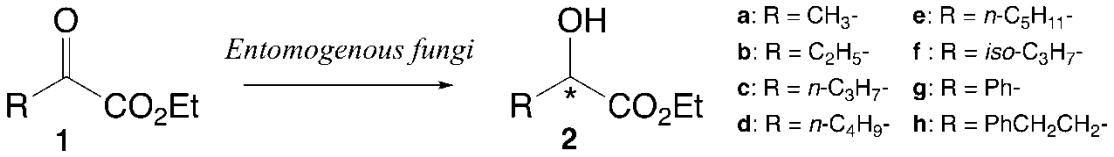
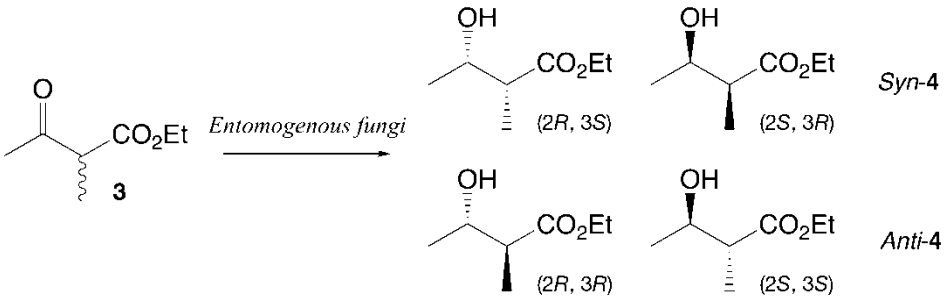


Figure.2 The reduction of ethyl 2-methyl-3-oxobutanoate (**3**) to ethyl 3-hydroxy-2-methylbutanoate(**4**) by entomogenous fungi



Therefore, in this study, an additive was introduced into the reaction mixture to improve the stereoselectivity of the product obtained from the reduction by the NBRC33061 strain (see Table 4).

In the reduction by the PDB-cultivated NBRC33061 strain, the addition of L-glutamate slightly improved the stereoselectivity of the produced hydroxy esters. On the other hand, the reduction of five substrates (**1a**, **1b**, **1c**, **1f**, and **1h**) in the presence of L-glutamate by the PGO-cultivated NBRC33061 strain yielded their corresponding (*S*)-alcohols with high conversion ratios and in excellent e.e.(>99% e.e.). It appears that the increase in reduced nicotinamide-adenine dinucleotide (possibly NADPH) derived from the oxidative degradation of L-glutamate accelerates the reduction of α-keto esters to the corresponding alcohols.

Reduction of *β*-Keto Esters by Tochukaso and Related Species

The ability of the wet cells of strains NBRC33061, 100684, 103832, and 109003 to reduce ethyl 2-methyl-3-oxobutanoate (**3**), a *β*-keto ester, was investigated (see Tables 5 and 6, Figure 2). Among the four strains cultured in PDB medium, NBRC33061 and 109003 reduced **3** to the corresponding *β*-hydroxy ester, with the *syn*/*anti* ratio of the product being predominantly the *anti*-form, and the stereoselectivity of the reduced carbonyl group at the 3-position being specific to the *S*-form (see Table 5).

In contrast, all four strains cultured in PGO medium were able to reduce **3**, but NBRC100684 and 103832 strains showed a conversion rate of less than 50% and a low *syn*/*anti* ratio. However, the reduction of the carbonyl group at the 3-position was stereospecific to the hydroxyl form of the *S*-form (see Table 6).

In strain NBRC109003, which showed a high *syn*/*anti* ratio of the reduced hydroxyester, the effect of additives on reduction was investigated to further improve the stereoselectivity of the produced alcohol (**4**) as shown in Table 7. In the reduction of **3**, the introduction of L-glutamate showed a higher conversion rate than the use of L-alanine.

In particular, the wet cells of strain NBRC109003 cultured in PGO medium performed the stereospecific reduction of 3 to the corresponding alcohol (**4**) with an excellent conversion rate of >99%, an e.e. of >99%, and a high *syn*/*anti* ratio. In this reaction, the substrate was reduced to only one of the four theoretically possible isomers; in other words, this microbial reduction reaction was able to produce a *β*-hydroxy ester having two chiral center carbons (see Figure 2).

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Author Contributions

Kohji Ishihara: Investigation, resources, formal analysis, writing-original draft preparation. Yu Takaki: Formal analysis, validation, writing-reviewing. Noriyoshi Masuoka: Data curation, supervision, writing-reviewing the final version of the manuscript. Kei Shimoda: Investigation, formal analysis, validation, writing- review and editing.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

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