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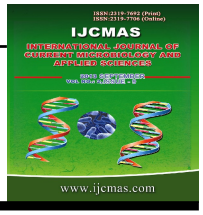

Original Research Article

Biocatalyst activity of entomogenous fungi: stereoselective reduction of carbonyl compounds using tochukaso and related species

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Keywords

Tohchukaso and related species; Entomogenous fungi; Biocatalyst; Stereoselective reduction; Chiral alcohol.

A B S T R A C T

To investigate the potential ability of tochukaso and related species to act as biocatalysts, we screened 13 entomogenous fungal strains. Two recommended media (potato-dextrose broth and 889 media) and a modified medium (PGO medium) were tested for liquid culture of these entomogenous fungi. Six strains (NBRC100741, 100742, 101754, 106941, 106945, and 106950) cultured using the PGO medium showed a good growth. The stereoselective reduction of α-keto esters and aromatic α-keto amide using these six strains was investigated. It was found that these strains possess a reducing activity toward various α-keto esters. Among them, the reduction of α-keto esters by *Isaria takamizusanesis* NBRC100741 in the presence of $_{L}$-glycine as an additive gave the corresponding α-hydroxy esters with a high conversion ratio and excellent enantioselectivity. Furthermore, it was found that the NBRC100742 strain reduced 2-chlorobenzoylforamide to (*R*)-2-chloromandelamide with excellent enantiomeric excess even at a high substrate concentration (75.0 mM). Thus, we found that tochukaso and related species have great potential to be used as biocatalysts for the stereoselective reduction of carbonyl compounds.

Introduction

A common concept is that, "medicines and foods have the same origin." Many centuries ago, fungi showing bioactivities such as medicinal properties have been recognized in Japan, Korea and China. The medicinal properties attributed to fungi include anticancer activity, antibiotic activity, antiviral activity, immune response-stimulating effects, anti-hypertensive effects, and blood lipid lowering effects (Wasser and Weis, 1999; Kaul, 2001). Therefore, pharmaceutical industries have shown great interest in novel compounds, extracted from the mycelium or fruiting body of fungi. For example, *Cordyceps*, a genus of ascomycete fungi (tochukaso in Japanese), are endoparasitoides, mainly on insect larvae, mature insects, and other arthropods (Holliday and Cleaver, 2008).

Cordyceps spp. have a long history as rare and exotic medicinal fungi. They have been highly regarded as cornerstones of traditional Chinese medicine (Mizuno, 1999; Coates *et al*., 2005). *Cordyceps* mushrooms have been used to treat various conditions, including respiratory and pulmonary diseases; renal, liver, and cardiovascular diseases; hyposexuality and hyperlipidemia. They are also used in the treatment of immune disorders and as adjuvant to modern cancer therapies (Holliday and Cleaver, 2008). Medicinal and pharmacological applications of *Cordyceps* mushrooms have been reported, while the possibility of its application in other fields of the mushroom is not well known. To isolate novel biocatalysts, we studied microbial production of useful substances such as optically active compounds by microorganisms and clarified the substance conversion abilities (especially, asymmetric reduction of carbonyl compounds) of yeasts, fungi, green algae, and bacteria such as 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). However, potential biocatalyst activities of entomogenous fungi like *Cordyceps* and their related species (such as *Elaphocordyceps*, *Ophiocordyceps*, *Torrubiella*, *Isaria*, *Nomuraea*) have not been investigated.

This study describes the stereoselective reduction of α-keto esters and their derivatives by tochukaso and related species (clavicipitaceae) as novel biocatalysts (Figure 1).

Instruments and Chemicals

Gas chromatography (GC) was performed using the GL Science GC-353 gas chromatographs (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), polypepton, olive oil, pectin (from apple), Daigo's potato dextrose agar (PDA), and trehalose were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Difco™ potato dextrose broth (PDB), Difco™ soluble starch, and Bacto™ yeast extract were purchased from Becton, Dickinson and Co., Franklin Lakes, NJ, USA. Peptone was purchased from Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan. Ethyl lactate (2a), ethyl 3-methyl-2-oxobutyrate (1f), ethyl 2-oxo-4-phenylbutyrate (1h), ethyl 2-hydroxy-4-phenylbutyrate (2h), and beef extract were purchased from Sigma-Aldrich. Ethyl benzoylformate (1g) and ethyl mandelate (2g) 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), 2-chlorobenzoylformamide (1i), 2-chloromandelamide (2i), and α-hydroxy esters (2b-f) were prepared according to the procedures described in the literature (Nakamura *et al*., 1998; Mitsuhashi and Yamamoto, 2005).

Microorganisms and Culture

Cordyceps militaris NBRC100741 (Japanese name: sanagi-take), *Cordyceps takaomontana* NBRC101754 (Japanese name: usukisanagi-take), *Conoideocrella luteorostrata* NBRC106950 (Japanese

name: hadanibeniiro - take), *Elaphocordyceps paradoxa* NBRC100945 (Japanese name: umemurasami-take), *Elaphocordyceps capitata* NBRC100997 (Japanese name: tanpo-take), *Elaphocordyceps ophioglossoides* NBRC100998 (Japanese name: hanayasuri-dake), *Hymenostilbe odonatae* NBRC106941 (Japanese name: yanma-take), *Isaria tenuipes* NBRC100694 (Japanese name: hanasanagi-take), *Isaria takamizusanesis* NBRC100742 (Japanese name: seminoharisenbon), *Nomuraea atypicola* NBRC106945 (Japanese name: kumo-take), *Ophiocordyceps sphecocephala* NBRC101752 (Japanese name: hachi-take), *Ophiocordyceps longissima* NBRC106965 (Japanese name: ezoharuzemi-take), and *Torrubiella superficialis* NBRC101755 (Japanese name kaigaramushikiirotsubu-take) were purchased from the National Institute of Technology and Evaluation, Biological Resource Center (NBRC, Japan). These clavicipitaceae strains were maintained at 25°C in PDA, while they were grown in PDB, 889 and a synthetic medium (PGO medium; 200 mL) for 6-15 days at 25°C with aerobic rotary shaking at 95 rpm in a baffled 500-mL flask in the dark. The PGO medium comprised 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).

The 889 medium (Tre-P-Y) comprised 20 g of trehalose, 3 g of peptone, and 1 g of Bacto™ yeast extract per liter of distilled water (pH 6.7). Entomogenous fungi cells were harvested by filtration on a filter paper (Whatman, No. 4) *in vacuo* and washed with saline (0.85% aqueous NaCl).

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

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 (reciprocating shaking at 120 rpm) at 25°C. 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 alcohols produced (Figure 1, 2a-i) was 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; 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 and at 175°C, 1i, 6.85 min; and 2i, 8.34 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) or Gamma DEX 225 capillary column (2f and 2i). The e.e. was calculated using the following formula: e.e. (%) = {(*R*-*S*)/(*R*+*S*)} x 100, where *R* and *S* are the respective peak areas in the GC analyses. The absolute configurations of the α-

hydroxy esters (2a-h) and aromatic α-hydroxy amide (2i) were identified by comparing their retention times from the GC analyses with those of authentic samples (Nakamura *et al*., 1998; Mitsuhashi and Yamamoto, 2005).

Results and Discussion

Screening of Entomogenous Fungi and Culture Media

To search for a suitable medium for the liquid culture, the amount of wet cells obtained by cultivating of the 13 entomogenous fungi in several culture media was measured. The recommended medium for most of the microorganisms tested in this study was PDA or 889 medium. However, all strains cultivated in the recommended medium resulted in 4.0 g or less of wet cells, even if cultured for over 3 weeks in PDB or 889 (Table 1). To improve the cultivation rate in PDB or 889 medium, our designed culture medium (PGO medium) was tested. We found that the cultivation in the PGO medium produced more wet cells than cultivation in other media. In particular, six strains (NBRC100741, 100742, 101754, 106941, 106945, and 106950) cultured in the PGO medium produced over 5.0 g of wet cells within 2 weeks. On the other hand, when NBRC106965 (hachi-take) and 101752 (ezoharuzemi-take) strains were cultured in the three media, it was not possible to obtain more than 2 g of wet cells, implying that these strains were not suitable as biocatalysts.

Therefore, we investigated the possibility that the six tochukaso and related species (NBRC100741, 100742, 101754, 106941, 106945, and 106950) cultivated in the PGO medium could act as biocatalysts for the asymmetric reduction of carbonyl compounds.

Reduction of α-Keto Esters by Tochukaso and Related Species

Six tohchukaso and related species (NBRC100741, 100742, 101754, 106941, 106945, and 106950) were tested for their ability to reduce α-keto esters (Figure 1). The results of the α-keto ester reductions are summarized in Table 2. We found that all the tochukaso and related species investigated in this study reduced α-keto esters (1a-h) to the corresponding α-hydroxy esters (2a-h). The reduction of α-keto esters by the NBRC100741, 100742, and 106945 strains exhibited a higher conversion ratio than the reduction by the NBRC101754, 106941, and 106950 strains; in particular, the NBRC100741and 100742 strains had high reducing activity for substrates having an alkyl chain (1a-d), but the stereoselectivity of the alcohols (2a-f) by reduced by the NBRC100741 showed low values. The stereoselectivity of the reduction product by the NBRC106945 and 100742 strains was higher than the reduction by the NBRC100741 strain. However, the selectivity was lower than 90% in the reduction of a substrate having a medium-to-long alkyl chain.

In the microbial reduction of carbonyl compounds using the common bakers' yeast or filamentous fungi, it is well known that introduction of small organic molecules or metal ions will increase stereoselectivity of the alcohols produced (Kawai *et al*., 1994; Kawai *et al*., 1995; Nakamura *et al*., 1996). In contrast, in the reduction using actinomycetes, there are several reports 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 several culture media.

NBRC	Scientific Name	PDB[1]	PGO[1]	889[1]
		Wet cells (g)[2]	Wet cells (g)[3]	Wet cells (g)[2]
100694	*Isaria tenuipes*	2.6	3.7	2.0
100741	*Cordyceps militaris*	1.7	6.4	1.1
100742	*Isaria takamizusanesis*	1.9	5.5	1.4
100945	*Elaphocordyceps paradoxa*	3.2	4.2	1.2
100997	*Elaphocordyceps capitata*	2.2	4.2	0.2
100998	*Elaphocordyceps ophioglossoides*	1.3	2.5	1.2
101752	*Ophiocordyceps sphaecocepala*	0.4	0.6	0.8
101754	*Cordyceps takaomontana*	2.5	7.6	0.8
101755	*Torrubiella superficialis*	1.0	3.7	0.1
106941	*Hymenostilbe odonatae*	3.5	5.3	3.4
106945	*Nomuraea atypicola*	3.5	6.1	3.9
106950	*Conoideocrella luteorostrata*	2.3	5.8	2.5
106965	*Ophiocordyceps longissima*	1.1	1.2	1.3

[1]Composition of each culture medium was described in materials and method section [2]The entomogenous fungi were grown in the medium at 25°C for 22 days with aerobic rotary shaking (100 min^{-1}) in baffled 500-mL flask in the dark condition. [3]The entomogenous fungi were grown in the medium at 25°C for 12 days with aerobic rotary shaking (100 min^{-1}) in baffled 500-mL flask in the dark condition.

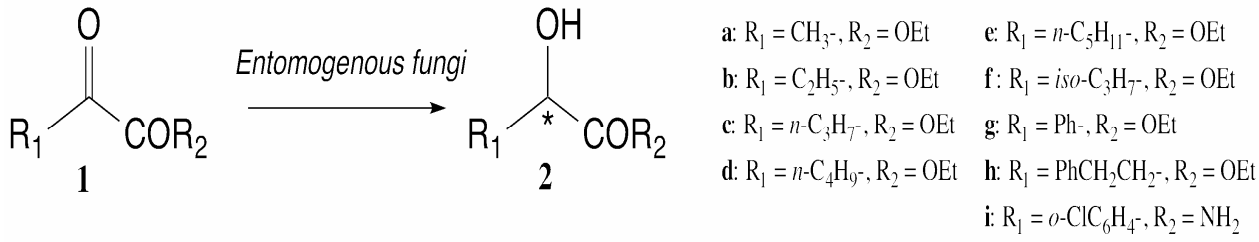

Figure 1. The reduction of α-keto esters (**1a-h**) and aromatic α-keto amide (**1i**) to corresponding alcohols (**2a-i**) by tochukaso and related species

Table.2 The reduction of α-keto esters (**1a-h**) and aromatic α-keto amide (**1i**) to the corresponding alcohols (**2a-i**) by tohchukaso and related species[1]

Product	*Cordyceps militaris* NBRC100741			*Isaria takamizusanesis* NBRC100742			*Cordyceps takaomontana* NBRC101754			*Hymenostilbe odonatae* NBRC106941			*Nomuraea atypicola* NBRC106945			*Conoideocrella luteorostrata* NBRC106950		
	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]	conv.[2] (%)	e.e.[3] (%)	(*R*/*S*)[3]
2a	>99	29	*S*	>99	99	*S*	>99	23	*R*	>99	45	*R*	>99	99	*R*	3	28	*R*
2b	>99	10	*R*	>99	96	*S*	61	74	*R*	>99	81	*R*	99	98	*R*	95	41	*R*
2c	>99	35	*R*	>99	85	*R*	26	25	*R*	84	15	*S*	98	63	*S*	70	2	*S*
2d	>99	28	*R*	>99	80	*R*	16	18	*R*	60	40	*S*	78	67	*S*	19	50	*S*
2e	44	10	*S*	>99	81	*S*	25	54	*R*	8	17	*R*	71	53	*S*	57	98	*S*
2f	>99	81	*R*	>99	87	*R*	>99	58	*R*	>99	61	*R*	>99	88	*R*	80	47	*R*
2g	65	>99	*R*	85	92	*R*	82	>99	*S*	85	81	*S*	88	44	*S*	90	46	*R*
2h	11	<1	*S*	68	83	*R*	5	20	*R*	7	>99	*S*	65	88	*S*	3	98	*R*
2i	96	97	*R*	>99	>99	*R*	91	94	*R*	94	99	*R*	>99	>99	*R*	98	98	*R*

[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 24 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 Effect of additive on the reduction of α-keto esters (**1a-h**) to the corresponding alcohols (**2a-h**) by tohchukaso and related species

Product	*Isaria takamizusanesis* NBRC100742 (Seminoharisenbon)						*Nomuraea atypicola* NBRC106945 (Kumo-take)					
	Additive: L-glycine			Additive: L -alanine			Additive: L -glycine			Additive: L -alanine		
	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*	>99	98	*S*	>99	98	*R*	>99	96	*R*
2b	>99	98	*S*	>99	99	*S*	>99	97	*R*	>99	94	*R*
2c	>99	93	*R*	>99	83	*R*	>99	86	*S*	96	71	*S*
2d	>99	90	*R*	98	77	*R*	96	79	*S*	85	81	*S*
2e	>99	94	*S*	98	91	*S*	98	88	*S*	72	84	*S*
2f	98	99	*R*	96	99	*R*	>99	>99	*R*	98	97	*R*
2g	>99	95	*R*	95	83	*R*	>99	75	*S*	93	94	*S*
2h	94	96	*R*	90	90	*R*	93	>99	*S*	90	90	*S*

[1]Substrate (0.15 mmol), 0.85% NaCl aq. (20 ml), and additive (7.5 mmol) were added to the wet cells (0.5 g) cultured in PGO medium, and the reaction mixture was incubated aerobically (reciprocating shaking at120 min^{-1}) at 25 °C for 24 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 Effect of substrate concentration on the reduction of aromatic α-keto amide (1i) to the corresponding hydroxy amide (2i) by tohchukaso and related species

Substrate (mM)	*I. takamizusanesis* NBRC100742			*N. atypicola* NBRC106945		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
7.5	>99	>99	*R*	>99	>99	*R*
22.5	>99	>99	*R*	>99	>99	*R*
37.5	>99	>99	*R*	98	>99	*R*
75.0	98	>99	*R*	96	98	*R*

[1]Substrate (0.15-1.50 mmol, dissolved in DMF), 0.85% NaCl aq. (20 ml) and additive 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.

Therefore, the effect of additives on the reduction of keto esters using 2 entomogenous fungi (NBRC100742 and 106945 strains) was investigated (see Table 3). Among the various additives used (e.g., allyl alcohol, methyl vinyl ketone, magnesium chloride, calcium chloride, glucose, fructose, sucrose, maltose, L-glycine, L-alanine, L-glutamate, and L-aspartate), the introduction of L-glycine and L-alanine increased the conversion ratio of the reduction. In particular, reduction by the NBRC100742 strain in the presence of glycine gave the corresponding α-hydroxy ester with a high conversion ratio (>94%) for all substrates. It appears that the increase of reduced nicotinamide- adenine dinucleotide (possibly NADPH) through the oxidative degradation of glycine accelerates the reduction of α-keto esters to the corresponding alcohols. Furthermore, following the introduction of L-glycine, stereoselectivity of the reduction by NBRC100742 also improved. However, improvement of the stereoselectivity following the addition of the small organic molecules or the metal salts that should significantly decrease the conversion ratio of the reduction was not also observed (data not shown).

Reduction of Aromatic α-Keto Amide by Tochukaso and Related Species

Six tohchukaso and related species were tested for their ability to reduce 2-chlorobenzoylforamide (**1i)**, an aromatic α-keto amide. As shown in Table 3, we found that the aromatic α-keto amide (**1i**) was reduced to the corresponding α-hydroxy amide (**2i**) by all the six strains. The conversion rate indicated a higher reduction power by the six strains tested, and it was found that the substrate was reduced to the corresponding α-hydroxy amide with high enantiomeric excess.

Among the strains used, the reduction by NBRC100742 and 106945 gave (*R*)-2-chloromandelamide (2i) with a high conversion ratio (>99%) and in excellent e.e. (>99%). Therefore, we investigated the effect of the substrate concentration on the reduction efficiency (see Table 4). In the reduction of 2-chlorobenzoylformamide (1i) by NBRC100742 strain, the reaction proceeded even at a high substrate concentration (75.0 mM) and gave (*R*)-2i with a high conversion ratio (98%) and in excellent stereo selectivity (>99% e.e.). These results have shown that the NBRC100742 and 106945 strains have a superior ability as biocatalysts, indicating the possibility of practical production of (*R*)-2-chloromandelamide.

Various α-keto esters and an aromatic α-keto amide were converted to the corresponding α-hydroxy esters by tohchukaso and related species. On the basis of the reduction conversion rates and the enantioselectivity of the products, we suggest that *Isaria takamizusanesis.*

NBRC100742 (seminoharisenbon) is a potential biocatalyst for the stereoselective reduction of keto esters and keto amide for obtaining the corresponding chiral hydroxy alcohols.

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