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Biocatalytic Ability of the Hairy Bracket Mushroom (*Trametes hirsuta*): Bio-reduction of Carbonyl Compounds

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A B S T R A C T

This study screened the potential biocatalytic abilities of four hairy bracket mushroom (*Trametes* strains) strains. Commercially available potato dextrose broth (PDB) and a medium recommended by the Fungus/Mushroom Resource and Research Center (MA medium) were used for culturing these mushrooms. Good growth was observed in two strains –TUFC13731 and 31398– cultured using the PDB. The stereoselective reduction of α- and β-keto esters by these strains was investigated, and they were found to reduce various α-keto esters. Specifically, the reduction of α-keto esters by the PDB-cultivated *T. hirsuta* TUFC13731 strain in the presence of L-glutamate as an additive yielded the corresponding α-hydroxy esters with a high conversion ratio and excellent enantioselectivity. Furthermore, this strain 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, hairy bracket mushrooms demonstrated great potential for application as biocatalysts for the stereoselective reduction of carbonyl compounds. The present study results also suggest that cryopreserved *T. hirsuta* cells can be used as biocatalysts for the asymmetric reduction of carbonyl compounds.

Introduction

Trametes hirsuta (Poliporaceae family; Japanese name: aragekawara-take), also known as the hairy bracket mushroom, is found globally. It is a saprophytic white-rot fungus that grows on dead hardwood trees and is 3–10 cm in diameter. Although inedible, it is a vital source of medicines and demonstrated pharmacological activities such as analgesic, anti-inflammatory, antispasmodic, and antibacterial properties (Begum *et al*., 2023). It can also decompose Remazol Brilliant Blue R (RBBR), a well-known model compound for studying the decomposition of persistent compounds and is a mushroom attracting attention in the fields of environmental science, such as for the removal of pollutants (Glazunova *et al*., 2024). Thus, the medicinal and environmental applications of *T.*

hirsuta have been reported. However, the possible applications of this mushroom in other fields have not been sufficiently explored. To discover novel biocatalysts, we have previously studied the microbial synthesis of useful products such as optically active compounds and elucidated the bioconversion abilities–especially, the asymmetric reduction of carbonyl compounds–of yeasts, fungi, green algae, and bacteria (actinomycetes) (Ishihara *et al.*, 1995; 2003; 2006; 2010; 2011b; 2013; 2015; 2017; 2019a; 2019b; 2022; 2024). We have also investigated the potential biocatalytic activities of edible mushrooms, including *Pleurotus salmoneostramineus*, *Ganoderma lucidum*, and *Flammulina velutipes,* etc., for the asymmetric reduction of carbonyl compounds, and identified several strains having an excellent reductive capacity (Ishihara *et al*., 2012). However, such investigations in other mushrooms are lacking.

This study describes the stereoselective reduction of α-keto esters and their derivatives by selected strains of hairy bracket mushroom acting as novel biocatalysts (Figures 1 and 2).

Materials and Methods

Instruments and Chemicals

Gas chromatography (GC) was performed using a GC-353 gas chromatograph (GL Science Inc., Tokyo, Japan) equipped with the capillary columns- DB-Wax, 0.25 □m, 0.25 mm x 30 m (Agilent Technologies, CA, USA); TC-1, 0.25 µm, 0.25 mm x 30 m (GL Science Inc.); CP-Chirasil-DEX CB, 0.25 µm, 0.25 mm x 25 m (Varian Inc., CA, USA); Gamma DEX 225, 0.25 µm, 0.25 mm x 30 m (Sigma-Aldrich Chemicals Inc., MO, USA). Ethyl pyruvate (Figure 1, **1a**), diatomaceous earth (granular) was purchased from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. Difco™ potato dextrose broth (PDB) and Difco™ malt 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 (Figures 2 and 3) were bought from the 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 following the procedures described previously (Nakamura *et al*., 1988).

Microorganisms and Culture

Four white rot fungi, *T. hirsuta* strains TUFC13731, 31398, 31400, and 31401 were used in this study. These strains were provided by the Fungus/Mushroom Resource and Research Center, (FMRC, Tottori University, Japan) through the National Bioresource Project (NBRP) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. The strains were cultured at 25 °C in an FMRC-recommended medium (MA medium) solidified with 2.0% (w/v) agar, while they were grown aerobically in 200 mL PDB or MA broth in a baffled 500 mL flask for 14 days at 25 °C in the dark, with rotary shaking at 120 rpm. The MA medium consisted of 20 g of Bacto™ malt extract/L of distilled water. The wet mushroom cells were harvested by passing through a Whatman No. 4 filter paper, *in vacuo*, and washed with 0.85% saline. For cryopreservation, after washing with saline, the fresh wet cells were frozen and stored at –20 °C for 3 weeks before use.

Reduction of α-and β-Keto Esters by Hairy Bracket Mushroom Cells

The saline-washed fresh wet cells (resting cells) or cryopreserved cells –0.5 g, dry weight approximately ~0.15 g–were resuspended in ϕ30 x 200 mm test tubes containing 20 mL of saline. Then, the substrate (0.15 mmol; 7.5 mM) was added, and the reaction mix was incubated aerobically at 25 °C under reciprocating shaking at 120 rpm. A 0.5 mL aliquot of the mixture was filtered using a short diatomaceous earth column (ϕ 10 x 30 mm), extracted with 5.0 mL diethyl ether, and then concentrated under reduced pressure.

Analysis

The bioconversion of the alcohols produced (Figure 1, **2a**–**h**) was measured using 1) 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 2) a TC-1 capillary column- 100 kPa He at 140 °C: **1h**, 10.02 min; and **2h**, 10.96 min. The enantiomeric excess (e.e.) of the product was ascertained on a GC instrument equipped with an optically active CP-Chirasil-DEX CB (**2a–e**, **2g**–

h, and **4**) or a Gamma DEX 225 (**2f**) capillary column. The following equation was applied for calculating the e.e.-

$$e.e.\ (\%) = ([R - S] / [R + S]) \times 100$$

where *R* and *S* are the respective peak areas of 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 using GC analyses with those of the authentic samples (Nakamura *et al*., 1988).

Results and Discussion

Screening of Mushroom Strains and Optimal Culture Media

The broth suitable for use in liquid culture was identified by measuring the amounts of wet cells obtained by cultivating the four *T. hirsuta* strains in PDB and MA media as shown in Table 1. In the MA medium, all four strains produced 1.4–3.6 g of wet cells. However, TUFC31400 and 31401 cultures in PDB yielded wet cell masses of <1 g. In contrast, TUFC13731 and 31398 produced >10 g wet cells, and TUFC13731 yielding at ~30 g wet cells. Therefore, we investigated the possibility of employing TUFC13731 and 31398 cultivated in the PDB medium as biocatalysts for the stereo selective reduction of carbonyl compounds.

Reduction of Keto Esters by Fresh Wet Cells of Hairy Bracket Mushroom

The two high-yielding strains–TUFC13731 and 31398–were tested for their capability to reduce α-keto esters (Figure 1). The results are summarized in Table 2. The two strains reduced α-keto esters to the corresponding α-hydroxy esters. Both demonstrated relatively high conversion rates with substrates having short alkyl side chains (ethyl pyruvate and ethyl 2-oxobutanoate), but showed low conversion rates (18%–44%) with substrates containing a long alkyl chain (ethyl 2-oxoheptanoate), a sterically bulky alkyl chain (ethyl 3-methyl-2-oxobutanoate), and an aromatic ring (ethyl benzoylformate). The stereoselective production of alcohols was higher with TUFC13731 than with TUFC31398, and the reduction of short alkyl chain substrates exhibited a high selectivity (>90% e.e. (*S*)). In particular, ethyl pyruvate (**1a**) and ethyl 3-methyl-2-oxobutanoate (**1f**) were reduced to the corresponding (*S*)-hydroxy esters (**2a** and **2f**, respectively) with stereospecificity (>99% e.e.). As shown in Table 3, both strains showed high conversion (>94%) and *syn*/*anti* ratios during the reduction of ethyl 2-methyl-3-oxobutanoate (**1h**), a b-keto esters. Specifically, TUFC13731 reduced the substrate to only one of the four theoretically possible isomers; i.e., this reaction synthesized a β-hydroxy ester having two chiral center carbons.

Reduction of Keto Esters by Cryopreserved Cells

If microorganisms can be frozen and preserved, even the wet cells of those with poor growth can be used, thereby expanding their utility as biocatalysts. Therefore, the abilities of cryopreserved cells of the two strains to reduce carbonyl compounds were investigated. The reductive conversion ratios for α- and β-keto esters declined slightly with cryopreserved cells compared to fresh wet cells, while the stereoselectivity of the products improved marginally (Tables 4 and 5). Therefore, the cells of the two *Trametes* strains can be utilized as biocatalysts even when cryopreserved. In our previous research, microorganisms with poor growth in broth were excluded from screening for applicability. However, as this study demonstrated the utility of cryopreserved cells, future efforts ca screen such microbes.

Table.1 The cultivation of four hairy bracket mushrooms in two culture media

Scientific name	TUFC No.	Wet cells (g / 200-mL of culture)	
		MA medium[1,2]	PDB medium[2]
Trametes hirsuta	13731	3.6	29
	31398	3.2	10
	31400	2.7	1.0
	31401	1.4	0.3

[1]Composition of MA medium was described in materials and method section.

[2]Four hairy bracket mushrooms were grown in the medium at 25°C for 14 days with aerobic rotary shaking (120 strokes/min) in a baffled 500-mL flask in the dark condition.

Table.2 The reduction of α-keto esters (**1**) to α-hydroxy esters (**2**) with the fresh wet cells of two *T. hirsuta* strains cultivated in PDB medium[1].

Product	*T. hirsta* TUFC 13731			*T. hirsta* TUFC 31398		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	79	>99	*S*	68	96	*S*
2b	73	96	*S*	53	92	*S*
2c	70	90	*S*	60	88	*S*
2d	62	82	*S*	32	72	*S*
2e	44	34	*R*	37	14	*R*
2f	25	>99	*S*	31	>99	*S*
2g	18	86	*R*	21	74	*R*

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the fresh wet cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) 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.

Figure.1 The reduction of α-keto esters (**1a–g**) to corresponding α-hydroxy esters (**2a–g**) by the hairy bracket mushroom (*Trametes hirsuta*)

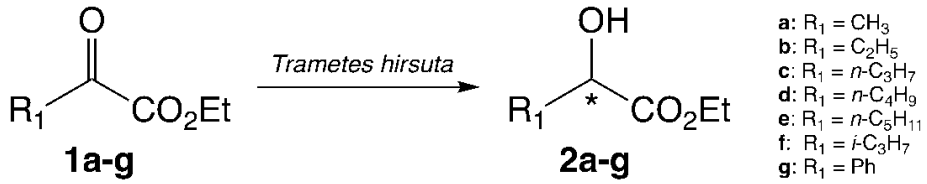

Figure.2 The reduction of ethyl 2-methyl-3-oxobutanoate (**1h**) to ethyl 3-hydroxy-2-methylbutanoate (**2h**) by the hairy bracket mushroom (*Trametes hirsuta*)

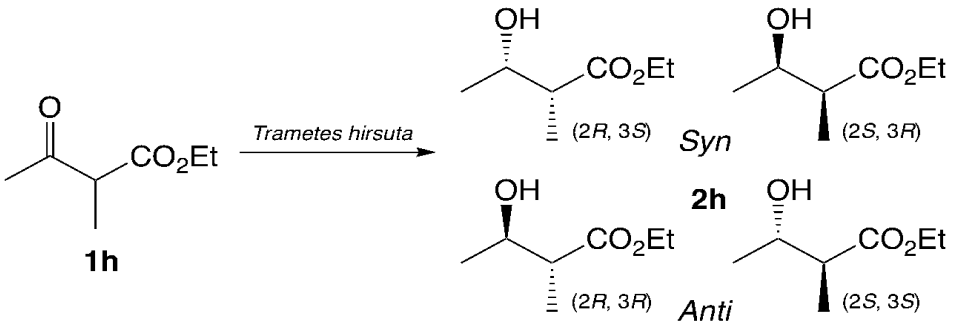

Table.3 The reduction of ethyl 2-methyl-3-oxobutanoate (1h) to ethyl 3-hydroxy-2-methylbutanoate (2h) with the fresh wet cells of two mushrooms cultivated in PDB medium[1].

TUFC No.	conv. (%)[2]	*Syn* / *Anti*[2]	e.e. (%)[3]	
			Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
13731	98	>99 / <1	>99	---[4]
31398	94	97 / 3	>99	>99

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

Table.4 The reduction of α-keto esters (1) to α-hydroxy esters (2) with the cryopreserved cells of two mushrooms cultivated in PDB medium[1].

Product	*T. hirsta* TUFC 13731			*T. hirsta* TUFC 31398		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	72	>99	*S*	47	98	*S*
2b	69	98	*S*	33	90	*S*
2c	68	92	*S*	20	90	*S*
2d	49	80	*S*	19	67	*S*
2e	31	49	*R*	21	25	*R*
2f	12	>99	*S*	13	>99	*S*
2g	10	78	*R*	11	80	*R*

[1]Substrate (0.15 mmol) and 0.85% NaCl aq. (20 ml) were added to the cryopreserved cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) 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 (1h) to ethyl 3-hydroxy-2-methylbutanoate (2h) with the cryopreserved cells of two mushrooms cultivated in PDB medium[1].

TUFC No.	conv. (%)[2]	*Syn* / *Anti*[2]	e.e. (%)[3]	
			Syn-(2*R*, 3*S*)	*Anti*-(2*S*, 3*S*)
13731	81	>99 / <1	>99	---[4]
31398	76	98 / 2	>99	>99

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

Table.6 The reduction of a-keto esters (1) to a-hydroxy esters (2) with the fresh wet cells of TUFC13731 strain cultivated in PDB medium in the presence of additive[1].

Product	L-Alanine			L-Glutamate		
	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]	conv. (%)[2]	e.e. (%)[3]	(*R*/*S*)[3]
2a	80	>99	*S*	96	>99	*S*
2b	72	94	*S*	92	98	*S*
2c	75	88	*S*	90	95	*S*
2d	60	84	*S*	82	90	*S*
2e	55	44	*R*	74	69	*R*
2f	34	>99	*S*	75	>99	*S*
2g	26	82	*R*	63	90	*R*

[1]Substrate (0.15 mmol), additive (5.0 mmol), and 0.85% NaCl aq. (20 ml) were added to the fresh wet cells (0.5 g) cultured in PDB medium, and the reaction mixture was incubated aerobically (reciprocating shaking at 120 strokes/min) 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.

Effects of Additives on the Reduction of α-Keto Esters by Fresh Wet Cells

The introduction of small molecular-weight organic molecules or metal ions during the microbial reduction of carbonyl compounds by bakers' yeast (*Saccharomyces cerevisiae*) or filamentous fungi increase the stereoselectivity of the alcohols produced (Kawai *et al*., 1994; Kawai *et al*., 1995; Nakamura *et al*., 1996). In contrast, while using microbes such as actinomycetes, basidiomycetes, entomogenous fungi, and microalgae, the addition of amino acids or sugars effectively improved the conversion rate and product stereoselectivity (Ishihara *et al*., 2003; 2010; 2011b; 2012; 2013; 2015; 2017; 2019a; 2019b; 2024). Therefore, in this study, the reaction mixture was introduced with an additive to enhance the stereoselectivity of the reduction product obtained while using TUFC13731 (Table 6). In general, reductases that use exogenous substrates, such as carbonyl compounds (e.g., keto esters), often require nicotinamide coenzymes (NADH or NADPH) as coenzymes (Kataoka *et al*., 1992; Nakamura *et al*., 1994; Yamaguchi *et al*., 2002; Ishihara *et al*., 2004a; 2004b; Qi *et al*., 2009; Ishihara *et al*., 2011a). Therefore, the effects induced by adding an amino acid that can be metabolized to produce these coenzymes were examined; such supplementation enhanced the reduction rate. In particular, L-glutamate increased the reduction rates for all substrates tested. Furthermore, the stereoselectivity of products obtained during the reduction of certain substrates was also improved. In particular, substrates 1a and 1f were stereospecifically reduced to the respective (*S*)-hydroxy esters.

In conclusion, two hairy bracket mushroom (*T. hirsuta*) strains, TUFC13731 and 31398 could convert α- and β-keto esters to corresponding hydroxy esters. Based on the conversion ratios and the stereoselectivity of the products, we suggest the culture of TUFC13731 in PDB for use as a potential biocatalyst during the stereoselective reduction of carbonyl compounds into chiral alcohols. In addition, the cryopreserved (freeze-stored) cells of this mushroom can be used as biocatalysts for the asymmetric reduction of carbonyl compounds.

Data availability

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

Author contributions

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

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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