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STEREOCHEMICAL CONTROL IN MICROBIAL REDUCTION 4. EFFECT OF CULTIVATION CONDITIONS ON THE REDUCTION OF β-KETO ESTERS BY METHYLOTROPHIC YEASTS.

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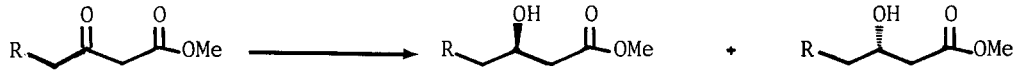
When β-keto esters are reduced by methanol grown yeast, drastic shift of the enantiomer excess of the products toward D-isomer formation is caused compared with those by glucose grown cells.

Control of the stereochemical course in biological transformation of organic compounds has recently attracted much attention of synthetic chemists.[1] Of various transformation, microbial reduction has been recognized as one of the most useful technique, and it is still a fascinating target of stereochemical control.[2,3] Such attempts for controlling microbial reduction can so far be classified into four groups. 1) Structural modification of substrates.[4,5] 2) Search for various microbes, *i.e.*, species, and/or even strains including some specific mutants.[6,7] Enzyme engineering will add new aspects in this category. 3) Use of (suicide) inhibitors against undesirable enzymes.[8] 4) Control of reaction condition such as substrate concentration. This includes cell immobilization technique recently developed by us.[9]

Another important factor which affects the stereochemical course, physiological state of cells, has, however, remained neglected. So, we now proceed to explore the fifth method, *i.e.*, extraction of cryptic ability of cells by enzyme induction[10] by changing cell growth conditions, which can be powerful but has not yet been successfully applied for yeast reduction in organic synthesis.

In the course of world-widely boiled studies on single cell protein and bioreactors, it has been found that many methylotrophs including "methanol" yeasts have characteristic secondary alcohol dehydrogenases in relatively large amount,[11-14] much interestingly for synthetic purpose. These dehydrogenases are usually induced only when cells are grown on methanol. Cells which have been used in microbial reduction are, however, only those grown on glucose or on other sugars such as sucrose even when "methanol" yeasts were used,[15] obviously (or at least possibly) failing to extract the

potentials. If we can exploit these dehydrogenases in whole intact cells as attempted for another kind of secondary alcohol dehydrogenase in thermophilic bacterium,[16,17] new method must be added within reach of organic chemists. To investigate such hopeful effects of change in growth condition on stereochemical course in yeast reduction, we chose a combination of some representative methylotrophic yeasts and β-keto ester reduction as an index reaction.



1a-c (D)-(-)-2a-c (L)-(+)-2a-c

a; R=H b; R=Me c; R=Cl

Table 1 Reduction of β-keto Esters by Methylotrophic Yeasts

Substrate	Yeast[a]	Growth Condition[b]	Major Product	e.e.[c] (%)	Chemical Yield (%)[d]
1a	*K. sp.*	G	(+)-2a	69	47
	K. sp.	M	(+)-2a	22	45
	H. poly.	G	(+)-2a	44	35
	H. poly.	M	(+)-2a	13	30
1b	*K. sp.*	G	(-)-2b	71	45
	K. sp.	M	(-)-2b	93	70
	H. poly.	G	(-)-2b	92	60
	H. poly.	M	(-)-2b	99	65
	BY		(-)-2b	54	47
1c	*K. sp.*	G	(-)-2c	42	50
	K. sp.	M	(-)-2c	68	20
	H. poly.	G	(-)-2c	74	12
	H. poly.	M	(-)-2c	90	5

[a] *K. sp.*; *Kroekera sp.* No. 2201 (AKU 4705). *H. poly.*; *Hansenula polymorpha* DL-1 (AKU 4327). BY; bakers' yeast (purchased from Oriental) [b] G; glucose grown. M; methanol grown. [c] Determined by GLC (OV-1701, 25 m, 180°C) (for a and b) or by 400MHz NMR (for c) analysis of their (+)-MTPA ester.[19] [d] Yields after preparative GLC. Relatively low yields of the products are due mainly to low conversion of the starting material (1a); about 20-30 % of 1a was usually recovered. In the case of 1c, chemical reaction of the compound with yeasts, which leads to enzyme inactivation (and trapping of the substrate), probably explains the low chemical yields of the products.

Kroekera sp. (*K. sp.*) No. 2201 (AKU 4705) and *Hansenula polymorpha* (*H. poly.*) DL-1 (AKU 4327) were grown for two days on a medium containing 20 ml of methanol, 4 g of NH_4Cl, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 2 g of yeast extract in 1 l of tap water.[18] Control cells were grown on the same medium except that 20 g of glucose was added instead of methanol as a carbon source. Each of 1 l culture gave more than 10 g of wet cells. Each reduction was performed as follows; To a fermenting suspension of 3 g of newly cultured wet cells in 30 ml of 10 % glucose solution, 300 mg of a substrate (final concentration; 10 g/l) was added and the mixture was incubated for 1 day at 30°C. Then, the mixture was filtered with the aid of celite powder, and the filtrate, combined with washings with water and with ether (each about 30 ml), was saturated with NaCl and extracted by 2 portions of 70 ml ether, dried, concentrated, and purified by preparative GLC. Enantiomer excess(e.e.) of each alcoholic product was determined by GLC or NMR analysis on the MTPA ester.[19] The sign of optical rotation was observed as $[\alpha]_D^{24}$.

As listed in Table 1, e.e. of the products turned out to shift commonly toward the formation of D-isomers irrespective of microbes and substrates employed, when methanol grown cells were used instead of glucose grown cells of the same yeasts. In particular, D-(-)-methyl-2-hydroxybutanoate (**2b**), a very important synthon in synthesizing natural products,[20,21] obtained by the reduction with methanol grown yeasts had unprecedentedly high e.e., clearly demonstrating the usefulness of the method. For the substrate (**1b**), ratios of apparent mean velosities to form each isomer, $\langle v_D \rangle / \langle v_L \rangle$, were estimated to be more than four to five fold enlarged. Furthermore, in the case of this compound, chemical yield was also improved by the method. This is likely to be attributable to the increase in absolute velosity of the reduction consistently with the induction of enzyme(s). Importantly, these results are very likely to indicate that *any kind of* methylotrophic yeast, provided grown on methanol, can be used to obtain (D)-**2b** in sufficiently high e.e., even if one can not obtain special strains used in the present study. Although it is still not clear if these results are caused by expected induction of secondary alcohol dehydrogenases or by some other factors such as enhancement of fatty acid synthetase (or elongation) system, such advantage of the approach will be applied for preparation not only of **2b** but also of various other important intermediates,[22-24] without immediate abandon of a microbe, in combination with other technique.[8,9]

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