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Effect of High Hydrostatic Pressure and High Pressure Homogenization on the Enantioselectivity of Microbial Reductions

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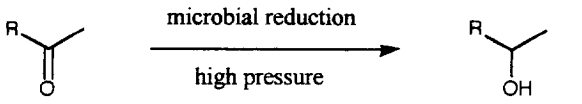
Abstract: The effect of high hydrostatic pressure and high pressure homogenization on the microbial reductions of the model prochiral ketones **1a-c** is described. Various strains of *Saccharomyces cerevisiae* and *Yarrowia lipolytica* are utilized in the reduction and higher enantioselectivities are generally achieved together with lower yields compared with the results obtained at atmospheric pressure. In some cases both increasing yields and inversion of enantioselectivity are reported. The effects of the hydrostatic and the homogenization pressure are also compared with the cell viability. Copyright © 1996 Elsevier Science Ltd

Since chiral alcohols are versatile and convenient building blocks in the synthesis of biologically important compounds, asymmetric reductions of ketones mediated by microrganisms are very useful methods for obtaining high chemical yields and high stereoselectivity.[1-4] On the other hand a microrganism does not necessarily afford the desired configuration, so various methods to control the bioreduction have been improved. The most common method is to screen microrganisms to find one suitable for a particular purpose,[5-7] the second is the modification of the substrate,[8-10] the third is the addition of an additive which affects the stereochemical course,[11-13] the fourth is to modify the reaction conditions.[14] Among the parameters that affect the course of a biotransformation, the pH,[15] the nature of the nutrients,[16] the cell immobilization,[14] the heat treatment[17] are well known.

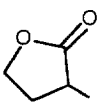
In this paper we describe the effect of high hydrostatic pressure and of high pressure homogenization on the reduction enantioselectivity of the model ketones **1a-c** with various yeast strains, e.g. *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Scheme).

The application of high hydrostatic pressure or high pressure homogenization to food processing are exciting newly developed "cold" technologies concerning improvement the food safety.[18-19] Pressure treatment can selectively inactivate the various microbial groups and, when used at sublethal doses, can induce morphological and physiological modifications to the microbial cells.[20-22] To our knowledge this is the first time that this technology has been applied to a microbial reduction of chemical compounds. For each

Scheme



1a-c **2a-b, 3c, 4c**

a; R = Ph **b**; R = CH_2=CH-CH_2-CH_2- **c**; R = 

biotransformation the survival cells are monitored. The level of survivors is expressed as log cfu (colony forming unit)/mL and calculated for each microrganism as the average between the data obtained for all three substrates at the same pressure. In Figure 1 the variation of the survival cells vs hydrostatic pressure applied to various strains is reported. The values on the ordinate axis correspond to the initial viable cell levels. The slopes of the various lines, log cfu *vs* pressure applied, indicate that *Saccharomyces cerevisiae* (strains ML31, ML29, RM1, RM97) is more resistant to the hydrostatic pressure than *Yarrowia lipolytica* (strains Y2, Y5, PO5, Y10). Minor differences between the two species are observed when the high homogenization pressures are used as indicated by Figure 2.

Figure 1

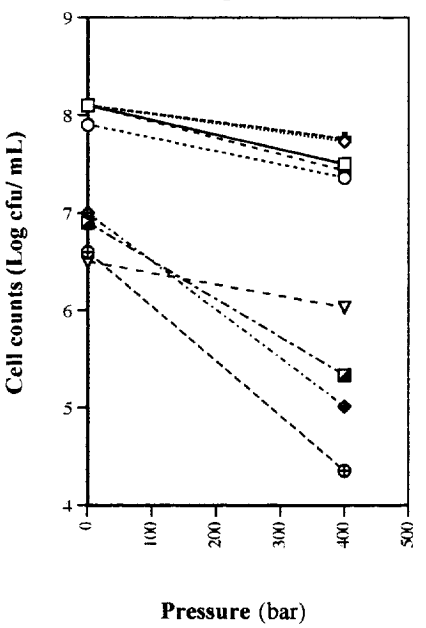


Figure 2

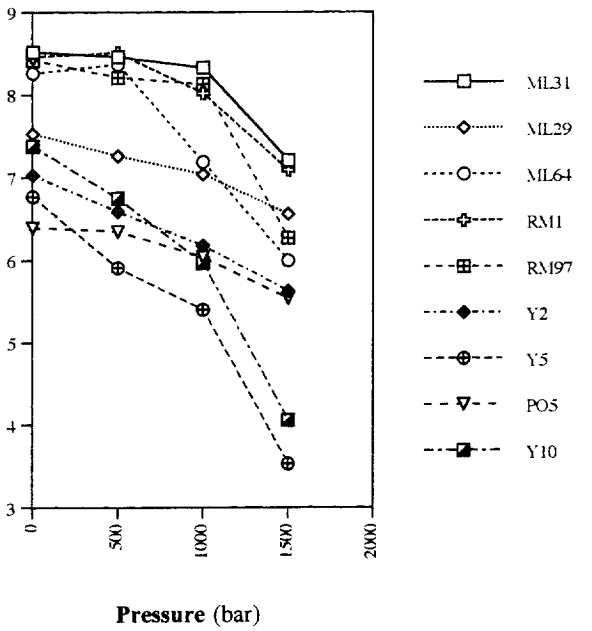


Figure 1-2. Effect of high hydrostatic pressure (fig. 1) and of high pressure homogenization (fig. 2) on cell viability of *Saccharomyces cerevisiae* and *Yarrowia lipolytica*.

The results of the microbial reductions of the acetophenone **1a**, subjected to hydrostatic pressure and pressure homogenization, compared with the biotransformations at atmospheric pressure are summarized in Table 1. The efficiency of the reduction does not depend on the survival of the cells either to the hydrostatic or homogenization pressure (Fig 1, 2). In fact, despite a general low loss of viability at least for *Saccharomyces cerevisiae* (Fig. 1), in all cases the microbial reductions of the acetophenone **1a** under 400 bar hydrostatic pressure afford very low yields of the alcohol **2a** (1-8%) in comparison with the blank experiment. On the other hand the microrganisms, subjected to the high pressure homogenization, produce generally the alcohol **2a** with lower yield according with the high loss of viability of the cells (Figure 2) but when the homogenization pressure increases up to 1000-1500 bar very high enantiomeric excesses (90-95%) of the *S*-alcohol **2a** (with *Sacchromyces cerevisiae*) and of the *R*-alcohol **2a** (with *Yarrowia lipolytica*) are obtained. Surprisingly *Saccharomyces cerevisiae* ML29 and ML64, after homogenization at 1500 bar, give higher yields (50 and 20%) with respect to the reduction at atmospheric pressure (24 and 2%) respectively, together with a substantial increase of enantioselectivity (for *S. c.* ML29 from 53% to 92%; for *S. c.* ML64 from 80% to 95%).

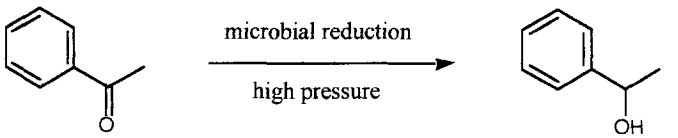
In Table 2 the results of the reduction of 5-hexen-2-one **1b** are reported. In this case also the effect of the hydrostatic pressure at 400 bar decreases highly the yields of the alcohol **2b**, while in the most of cases the process of homogenization affects scarcely them. On the other hand the reduction with *Saccharomyces cerevisiae* strains gives the S-alcohol **2b** with higher enantiomeric excesses with respect to the reduction at atmospheric pressure. The best results have been obtained at 500 bar homogenization with *S. cerevisiae* RM1 (81% of **2b**, 93% ee of the S-enantiomer) and at 1500 bar with *S. cerevisiae* RM97 (20% of **2b**, 95% ee of S-enantiomer). On the contrary *Yarrowia lipolytica* Y2 and PO5 afford, increasing the pressure, the alcohol **2b** with comparable yield to the reduction at atmospheric pressure while the enantiomeric excesses gradually decreased eventually to invert the configuration from the R-enantiomer to the S-enantiomer.

As reported for ketone **1b**, the yields of microbial reductions of the ketone **1c** are scarcely influenced by high pressure homogenization with respect to the reduction at atmospheric pressure while the yields of the reactions carried out under hydrostatic pressure are normally much lower (see Table 3).

In all cases the microbial reductions of **1c**, after high pressure homogenization, afford very good yields of enantiomerically pure *syn*-**3c** (100% of 3R,1'R-enantiomer), comparable with those obtained at atmospheric pressure. The only exception to this behaviour is given by *Yarrowia lipolytica* Y5 which produces at atmospheric pressure the alcohol **3c** with very poor yield (6%) while, increasing the homogenization pressure, affords mounting yields up to 91% at 1500 bar. On the other hand *Saccharomyces cerevisiae* RM1, in contrast with other microrganisms, gives the *syn*-alcohol **3c** with the opposite configuration (3S,1'S-enantiomer) with not so good ees (55% at atm. pressure, 19% at 400 bar and 56-60% after homogenization) together with the *anti*-alcohol **4c** (ee 100% of 3R,1'S-enantiomer). The yields are practically unaffected by homogenization at 500 and 1000 bar while a low decrease is achieved at 1500 bar. As usual the effect of hydrostatic pressure give rise to lower yields of the syn-alcohol **3c**.

In conclusion, in all cases the effect of the pressure (both hydrostatic or homogenization) decreases the cell viability although in different way. The hydrostatic (400 bar) pressure does not practically affect the viability of the *Saccharomyces cerevisiae* strains while it is deadly to *Yarrowia lipolytica* cells. Although recent publications have given new information on the inactivation kinetics of microrganisms and spores,[23] more data are necessary to understand and control the effects of high pressure on cells and biomolecules.

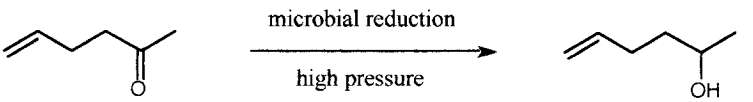
Table 1. Effect of the presssure on microbial reduction of ketone **1a**.



1a **2a**

microrganism	pressure	yield %	ee% (abs. conf.)
S. cerev. ML31	atm.	**2a** (42)	84 (S)
	hydrostatic 400 bar	**2a** (8)	84 (S)
	homogen. 500 bar	**2a** (45)	90 (S)
	homogen. 1000 bar	**2a** (44)	92 (S)
	homogen. 1500	**2a** (24)	93 (S)
S. cerev. ML29	atm.	**2a** (24)	53 (S)
	hydrostatic 400 bar	**2a** (6)	70 (S)
	homogen. 500 bar	**2a** (23)	78 (S)
	homogen. 1000 bar	**2a** (39)	81 (S)
	homogen. 1500 bar	**2a** (50)	92 (S)
S. cerev. ML64	atm.	**2a** (2)	80 (S)
	hydrostatic 400 bar	**2a** (1)	100 (S)
	homogen. 500 bar	**2a** (2)	80 (S)
	homogen. 1000 bar	**2a** (9)	88 (S)
	homogen. 1500 bar	**2a** (20)	95 (S)
Yarr. lip. Y5	atm.	**2a** (31)	57(R)
	hydrostatic 400 bar	**2a** (1)	100(R)
	homogen. 500 bar	**2a** (8)	75 (R)
	homogen. 1000 bar	**2a** (5)	60 (R)
	homogen. 1500 bar	**2a** (4)	95 (R)
Yarr. lip. Y10	atm.	**2a** (44)	80 (R)
	hydrostatic 400 bar	**2a** (2)	100 (R)
	homogen. 500 bar	**2a** (22)	80 (R)
	homogen. 1000 bar	**2a** (10)	95 (R)
	homogen 1500 bar	**2a** (5)	95 (R)

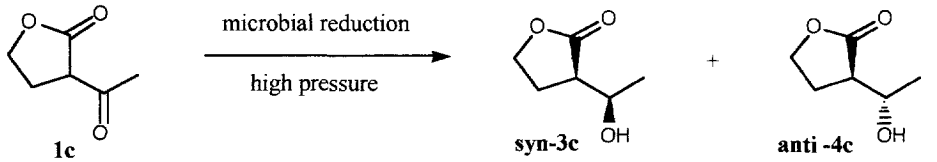
Table 2. Effect of the pressure on microbial reduction of ketone **1b**.



1b **2b**

microrganism	pressure	yield %	ee% (abs. conf.)
S. cerev. RM1	atm.	**2b** (77)	84 (S)
	hydrostatic 400 bar	**2b** (21)	100 (S)
	homogen. 500 bar	**2b** (81)	93 (S)
	homogen. 1000 bar	**2b** (47)	76 (S)
	homogen. 1500	**2b** (41)	95 (S)
S. cerev. RM97	atm.	**2b** (26)	71 (S)
	hydrostatic 400 bar	**2b** (19)	77 (S)
	homogen. 500 bar	**2b** (23)	80 (S)
	homogen. 1000 bar	**2b** (26)	74 (S)
	homogen. 1500 bar	**2b** (20)	95 (S)
S. cerev. ML29	atm.	**2b** (81)	27 (S)
	hydrostatic 400 bar	**2b** (17)	0
	homogen. 500 bar	**2b** (71)	27 (S)
	homogen. 1000 bar	**2b** (41)	17 (S)
	homogen. 1500 bar	**2b** (66)	35 (S)
Yarr. lip. Y2	atm.	**2b** (88)	16 (R)
	hydrostatic 400 bar	**2b** (4)	23 (S)
	homogen. 500 bar	**2b** (91)	8 (R)
	homogen. 1000 bar	**2b** (89)	0
	homogen. 1500 bar	**2b** (86)	11 (S)
Yarr. lip. PO5	atm.	**2b** (22)	14 (R)
	hydrostatic 400 bar	**2b**(1)	20 (R)
	homogen. 500 bar	**2b** (14)	7 (S)
	homogen. 1000 bar	**2b**(25)	33(S)
	homogen 1500 bar	**2b**(13)	38 (S)

Table 3. The effect of the pressure on microbial reduction of the ketone **1c**.



microrganism	pressure	product **3**		product **4**	
		yield %	ee%(abs. conf.)	yield%	ee(abs.conf.)
S. cerev. ML31	atm.	61	100 (3R,1'R)		
	hydrostatic 400 bar	45	100 (3R,1'R)		
	homogen. 500 bar	**73**	**100 (3R,1'R)**		
	homogen. 1000 bar	68	100 (3R,1'R)		
	homogen. 1500	66	100 (3R,1'R)		
S. cerev. RM1	atm.	50	55 (3S,1'S)	49	100 (3R,1'S)
	hydrostatic 400 bar	27	19 (3S,1'S)	4	100 (3R,1'S)
	homogen. 500 bar	48	56 (3S,1'S)	48	100 (3R,1'S)
	homogen. 1000 bar	48	63 (3S,1'S)	50	100 (3R,1'S)
	homogen. 1500 bar	35	60 (3S,1'S)	30	100 (3R,1'S)
S. cerev. ML29	atm.	98	100 (3R,1'R)		
	hydrostatic 400 bar	34	100 (3R,1'R)		
	homogen. 500 bar	98	100 (3R,1'R)		
	homogen. 1000 bar	97	100 (3R,1'R)		
	homogen. 1500 bar	98	100 (3R,1'R)		
Yarr. lip. Y5	atm.	6	100 (3R,1'R)		
	hydrostatic 400 bar	6	100 (3R,1'R)		
	homogen. 500 bar	25	100 (3R,1'R)		
	homogen. 1000 bar	64	100 (3R,1'R)		
	homogen. 1500 bar	**91**	**100 (3R,1'R)**		
Yarr. lip. Y10	atm.	74	100 (3R,1'R)		
	hydrostatic 400 bar	10	100 (3R,1'R)		
	homogen. 500 bar	53	100 (3R,1'R)		
	homogen. 1000 bar	**94**	**100 (3R,1'R)**		
	homogen 1500 bar	43	100 (3R,1'R)		

Moreover, the action mechanism of the two treatments is different. In contrast with the hydrostatic pressure, in the high homogenization pressure treatment the fluid is forced through a narrow gap and undergoes a rapid acceleration after which there is an extreme drop in pressure to which can be attributed the major role in the microbial cell deactivation.

Hydrostatic pressure effects on chemical reactions are usually interpreted in terms of intrinsic and solvent contributions.[24] In general high pressure stabilizes hydrogen bonds, induces reversible changes such as dissociation of protein-protein complexes, the binding of ligands and conformational changes.[25]
It has been reported that hydrostatic pressure negatively affects the reactions leading to a volume increase and favours the reaction leading to a volume decrease.[26]

The low reduction yield obtained under hydrostatic pressure, with respect to the control, could be attributed to this effect. On the other hand the promising results observed when the cell suspension was instantaneously processed with the homogenization can be interpreted as depending on various factors including an activation on the hydrogenase activity, due to a conformational changes, and a "de novo" synthesis of the enzymatic proteins. This last phenomenon could be associated to a production of the so called heat stress proteins (HSPs) induced in the microbial cells by various chemico-physical challenges.[27]
In order to improve the promising results obtained regarding the enantiomeric excesses and the inversion of configuration of the alcohol **2b**, obtained with *Yarrowia lipolytica*, a more detailed knowledge of the modification of the enzyme protein and of its interaction with the reaction substrate, also in relation to cell viability, is necessary.

Experimental

General. For the high hydrostatic pressure treatment an instrument specially made for DPVA (Dipartimento di Protezione e Valorizzazzione Agroalimentare) by Tecnopressa (Milan) was employed. The transmission liquid consisted of water flowing into a 2 liter chamber by means of a manual pump system. The maximum pressure attained was 400 bar. For the high pressure homogenization a homogenizer from Nio Soavi (Parma, Italy) OBL 20 having a capacity of 0-30 L and a 'PA'NS valve was used for treatments of cells cultures. For the cell viability Saboraud Agar was used. [20]

Enantiomer separation has been made on Megadex 5 column (25 X 0.25 mm) containing dimethyl-*n*-pentyl-β-cyclodextrine in OV 1701 from Mega s.n.c.: carrier gas: helium (0.8 atm). For reduction of **1a**: temp.130-200°C (2°C/min); retention time in min: **1a** 5.32, (*R*)-**2a** 7.11, (*S*)-**2a** 7.32. For reduction of **1b**: temp. 80-150°C (0.5°C/min); retention time in min: **1b** 4.59, (*S*)-**2b** 7.87, (*R*)-**2b** 8.06. For reduction of **1c**: temp. 100-200°C (0.5°C/min); retention time in min: **1c** 16.69, the *syn* and *anti*-alcohols as trifluoroacetyl derivative (3*R*,1'*R*)-**3c** 23.33, (3*S*,1'*S*)-**3c** 23.97, (3*S*,1'*R*)-**4c** 21.95, (3*R*,1'*S*)-**4c** 22.49.

The yeasts used for the microbial reduction at high pressure belong to DPVA collection and are the following : *Saccharomyces cerevisiae* ML29, ML31, ML64, RM1, RM35, RM72, RM97, 633, 635 and *Yarrowia lipolytica* Y2, Y5, Y9, Y10, PO5, PO19.

The ketones **1a-c** are commercially available. The alcohol **2a-b**, **3c** and **4c** has been characterized in a previous work. [28]

Microbial reductions of ketones 1a-c under high hydrostatic pressure. General procedure. A synthetic culture medium is prepared adding to 1 L of water glucose (50 g), $(NH_4)_2SO_4$ (5 g), KH_2PO_4 (2 g), $CaCl_2$ (0.25 g), $MgSO_4.7H_2O$ (0.25 g), inositol (25 mg), H_3BO_3 (1 mg), $ZnSO_4$ (1 mg), $MnCl_2$ (1 mg), $FeCl_2$ (0.5 mg), $CuSO_4$ (0.1 mg), tiamine (0.3 mg), biotin (0.025 mg), calcium pantothenate (0.3 mg), pyridoxine (0.3 mg), and nicotinic acid (0.3 mg). A cell suspension of the selected yeast was inoculated in 10 mL of the sterilized synthetic medium and grown at 27°C for 48 h on a reciprocatory shaker. The grown culture (1 mL) was set up in 50 mL conical flask containing the synthetic medium (35 mL) together with small amounts of the selected ketone **1** (70 µL of a solution prepared dissolving 0.1 g of the substrate in 1 mL of DMF). After growing for 48 h, a further 280 µl of the substrate solution was added and the cell culture was poured in a aluminium bag previuosly sterilized with H_2O_2 and washed with sterilized water. The bag was sealed hermetically with heat, poured into the hydrostatic chamber, the pressure was manually going up to 400 bar. The incubation under hydrostatic pressure was continued for 48 h at room temperature. Parallely a blank experiment of microbial reduction was carried out at atmospheric pressure starting from the same cell culture utilized for the reduction at 400 bar. When the reaction was stopped the cell viability of each microrganism was determined. The data are summarized in Figure 1. The suspension is removed by centrifugation, the mixture is extracted with diethyl ether and dried over anhydrous Na_2SO_4. The crude reaction products are analyzed by GLC on a chiral column. The results for each substrate are summarized in the Tables 1-3.

Microbial reductions of ketones 1a-c after high pressure homogenization. General procedure. A cell suspension of the selected yeast was inoculated in 10 mL of the sterilized synthetic medium (see above) and grown at 27°C for 48 h on a reciprocatory shaker. The grown culture (10 mL) was set up in 500 mL conical flask containing the synthetic medium (350 mL) together with small amounts of the selected ketone **1** (0.7 mL of a solution prepared dissolving 0.1 g of the substrate in 1 mL of DMF). After growing for 48 h, a further 2.8 ml of the substrate solution was added and the cell culture was poured in the high pressure homogenizer. During the process the reaction mixture is forced under pressure through a narrow gap where it is subjected to rapid acceleration after which it undergoes an extreme drop in pressure. After homogenization at 1 (blank experiment), 500, 1000, and 1500 bar, the cell cultures (50 mL for each fraction) were collected in 100 mL conical flask and poured in a reciprocatory shaker for 48 h at 27°C. When the reaction is stopped, the cell viability was determined for each fraction. The data are summarized in Figure 2. The suspension is removed by centrifugation, the mixture is extracted with diethyl ether and dried over anhydrous Na_2SO_4. The crude reaction products are analyzed by GLC on a chiral column. The results for each substrate are summarized and compared with those at hydrostatic pressure in the Tables 1-3.

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