

J. Haberland · A. Kriegesmann · E. Wolfram
W. Hummel · A. Liese

Diastereoselective synthesis of optically active (2*R*,5*R*)-hexanediol

Received: 17 October 2001 / Revised: 18 December 2001 / Accepted: 20 December 2001 / Published online: 8 February 2002
© Springer-Verlag 2002

Abstract Diastereoselective reduction of diketones with *Lactobacillus kefir* DSM 20587 was examined. The reduction of both oxo-functions proceeded highly diastereoselectively. (2*R*,5*R*)-Hexanediol **3** was produced starting from (2,5)-hexanedione **1** in quantitative yields with enantiomeric excess >99% and diastereomeric excess >99%. The reaction conditions were optimized: maximum yield of (2*R*,5*R*)-hexanediol was reached at pH 6, 30°C and with equal amounts of substrate and cosubstrate. The applicability of the system in fed-batch experiments was demonstrated. The feed specific biomass concentration required to reach maximal yield and selectivity in fed-batch mode was determined.

Introduction

Enantiopure diols are important building blocks for fine chemicals, particularly pharmaceuticals and agrochemicals (Ager 1999). γ -Diols are, for example, the main building blocks of chiral ligands like DuPhos or 1,2-bis(phospholano)ethane (BPE; Burk et al. 1995, 1996). Enantiopure alcohols can either be produced by kinetic resolution or by enantioselective reduction of the corresponding prochiral oxo-function (Liese et al. 2000). In the former, a maximal yield of 50% is possible if a catalyst with 100% selectivity is applied. In the latter, 100% yield is possible with a selective catalyst. Currently, (2*S*,5*S*)- and (2*R*,5*R*)-hexanediol are synthesized on an industrial scale by lipase-catalyzed transesterification starting from the racemic/meso mixture (2,5)-hexanediol (Taylor et al. 2000). Using this method, the theoretical

yields are 25% for (2*S*,5*S*)-hexanediol and 75% for (2*R*,5*R*)-hexanediol after subsequent chemical inversion of the (*R*,*S*)-monoester and downstream processing.

There are many ways to produce the (*S*,*S*)-enantiomers, including yeast reductions (Bertau and Bürli 2000; Bertau 2001) and reductions with, for example, horse liver alcohol dehydrogenase (ADH). High yields and high enantioselectivities for the synthesis of (*R*)-alcohols have been reported using ADH isolated from *Lactobacillus kefir* (Hummel 1990; Bradshaw et al. 1992; Wolberg et al. 2000). The *L. kefir* ADH is NADPH-dependent and there is always the problem of finding a suitable way of recycling this cofactor.

To overcome this problem, resting whole cells of *L. kefir* can be used. Starting from (2,5)-hexanedione, both oxofunctions are reduced to (2*R*,5*R*)-hexanediol in quantitative yields with enantio- and diastereo selectivities of >99% (Hummel et al. 1999). The reduction equivalents are all supplied internally in the form of nicotinamide cofactors. These are regenerated by the metabolism of the whole cell utilizing glucose.

To our knowledge, there is only one other possibility known for producing (*R*,*R*)-diols with whole cell biotransformations (Ikeda 1996); resting cells from *Pichia farinosa* IAM 4682 can also be used for the bioreduction shown in Fig. 1. A yield of only 83% and a diastereomeric excess of 95% are achieved by this method. The specific diketone-reducing activity of this strain is much lower than that of *L. kefir*.

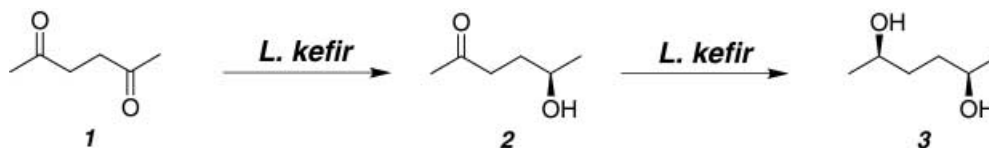
Besides this biological method of synthesizing (2*R*,5*R*)-hexanediol or other enantiopure diols, there are also some chemical methods that lead to these products (Caron and Kazlauskas 1994). The chiral ligands mentioned above – as part of, for example, Wilkinson catalysts – offer the possibility of reducing prochiral oxo-functions (Burk et al. 1991). Some other ligands, such as 2,2'-bis(diphenylphosphino)-1,1'-binaphthyl (BINAP; Akutagawa 1995), can also be used in transition metal-based catalysts.

Here, we present an efficient whole cell biotransformation, utilizing glucose as a cheap reduction equivalent

J. Haberland · A. Kriegesmann · E. Wolfram · A. Liese (✉)
Forschungszentrum Jülich GmbH, Institute of Biotechnology,
52425 Jülich, Germany
e-mail: a.liese@fz-juelich.de
Tel.: +49-2461-616044, Fax: +49-2461-613870

W. Hummel
Heinrich-Heine Universität Düsseldorf,
Institute of Enzyme Technology,
52426 Jülich, Germany

Fig. 1 Diastereoselective reduction of **1** (2,5)-hexanedione to **3** (2*R*,5*R*)-hexanediol with resting whole cells of *Lactobacillus kefir*



lent, to produce (2*R*,5*R*)-hexanediol in quantitative yields with >99% enantio- and diastereo selectivity.

Materials and methods

Chemicals

All chemicals used were purchased from Sigma, Fluka (Neu Ulm, Germany). Medium ingredients for the fermentation of *L. kefir* were purchased from Merck (Darmstadt, Germany).

Cells

L. kefir DSM 20587 was cultivated on MRS medium: 10 g/L casein peptone, 5 g/L sodium acetate, 10 g/L meat extract, 2 g/L K_2HPO_4 , 5 g/L yeast extract, 22 g/L glucose- H_2O , 1 g/L Tween 80, 2 g/L diammonium hydrogencitrate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot H_2O$ at a starting pH of 6.5 in 100 mL cultures. After 2 days of culture at 30°C on a reciprocal shaker set to 60 rpm, five 100 mL cultures were added to a 10 L glass fermentor containing 9.5 L of MRS medium. The fermentor was stirred for 2 days at 30°C and 100 rpm. After finishing this intermediate culture the whole suspension was transferred into a 300 L fermentor (Chemap, Volketswil, Switzerland) containing 190 L MRS-medium. After a further 2 days of cultivation at 30°C (300 rpm), an OD_{660nm} of 4 was reached and the cells were harvested with a cell separator (Typ SA 1, Westfalia Separator, Oelde, Germany) and stored at -20°C.

General methods

Cells (6 g) stored at -20°C were thawed, resuspended in 0.9% NaCl solution and centrifuged at 5,000 rpm for 15 min. The supernatant was discarded. Glucose was added first to the reaction vessel at a temperature of 30°C. The cells were then resuspended in 60 mL bidistilled water and flushed into the reaction vessel. The pH value was adjusted to 6 by adding 4 N NaOH. The reaction was started by adding the substrate: (2,5)-hexanedione. Samples (500 μ L) were withdrawn and cells were separated using a centrifuge (2 min, 14,000 rpm). The pellet was discarded and the supernatant stored at -20°C before analysis.

Fed-batch with *L. kefir*

The starting volume of every fed-batch experiment was 100 mL. As feed solutions, 2 M (2,5)-hexanedione and 2 M glucose in bidistilled water were used. Both solutions were fed with an autotitrator (Dosimat 665; Metrohm, Herisau, Switzerland). The feed rates were 2.4 mmol/h, 7.2 mmol/h, and 24 mmol/h for the glucose solution and 2.4 mmol/h, 7.2 mmol/h, 24 mmol/h for the (2,5)-hexanedione solution. Four batches were carried out in parallel. The feed rates were controlled by the "Fed-batch pro"-system (DASGIP, Jülich, Germany).

Analyses

Optical densities were measured using a Shimadzu UV-160 spectrophotometer. Quantification of (2,5)-hexanedione (1), 5-hydroxy-

hexane-2-one (2) and (2,5)-hexanediol (3) was carried out using an Agilent HP-5890A gas chromatograph with a Permabond Carbowax 20 M column (50m \times 0.32 mm ID, Macherey-Nagel, Düren, Germany), at 210°C with a flame ionization detector and helium as carrier gas (1.5 bar). To minimize injection error, (1,5)-pentanediol (4) was used as an internal standard. Retention times were: (1) 4.36 min, (2) 4.89 min, (3) 5.82 min, and (4) 8.18 min.

To determine the enantio- and diastereo selectivity of all diastereomers, the samples analyzed had to be derivatized with trifluoroacetic anhydride prior to analysis as follows. Aqueous sample (300 μ L) was extracted twice with 300 μ L $CHCl_3$. Trifluoroacetic acid anhydride (100 μ L) was added to the organic phase and incubated at 70°C for 30 min. The sample was evaporated to dryness and redissolved in 500 μ L $CHCl_3$.

Determination of the diastereomeric excess was performed on an HP-6890 gas chromatograph with a β -I/P-Cyclodex column (Chromatographie-Service, Langerwehe, Germany), at 70°C with a flame ionization detector and hydrogen as carrier gas (1 bar). Retention times were: (*R,R*)-(3) 21.45 min, (*S,S*)-(3) 22.03 min, meso-(3) 25.05 min.

Glucose and lactate were quantified by HPLC (Jasco) with a Aminex HPX-87H column (300 mm \times 7.5 mm ID, Bio-Rad, Munich, Germany). A diode array detector for lactate and an evaporate light scattering detector (evaporator 120°C, nebuliser 90°C, N_2 0.7 L min^{-1} , Polymer Laboratories, Darmstadt, Germany) for glucose were used. As eluent, an aqueous solution of 0.5% trifluoroacetic acid with a flow of 0.5 mL min^{-1} was used. The retention times were: glucose 10.8 min, lactate 14.9 min.

Results

General

Resting cells of *L. kefir* DSM 20587 catalyzed the reduction of (2,5)-hexanedione to (2*R*,5*R*)-hexanediol (Fig. 1) in a whole cell biotransformation. In batch experiments, quantitative yields and enantio- and diastereo-selectivities of >99% were achieved.

Glucose was added as a cosubstrate; without adding cosubstrate, no reaction was observed. The cosubstrate is necessary to drive the internal cofactor regeneration cycles in the cell for the NADPH-dependent ADH. *L. kefir* is known from the literature to be heterofermentative (Kandler and Kunath 1983), meaning that 1 mol lactic acid is formed per mol glucose (Fig. 2). Because of this, the pH shift during the course of the reaction is very large.

To circumvent the use of high amounts of buffer salts, which would need to be removed during downstream processing, the pH is titrated with an autotitrator (4 N NaOH). The optimal pH was determined as 6 (Fig. 3). At this pH value, the selectivity towards (2*R*,5*R*)-hexanediol reached 100% after 2 h in batch experiments. The selectivity used in all figures is the ratio of product to the sum of product and intermediate, and is defined as:

$$\text{selectivity} = \frac{c_{\text{product}}}{c_{\text{product}} + c_{\text{intermediate}}} \quad (1)$$

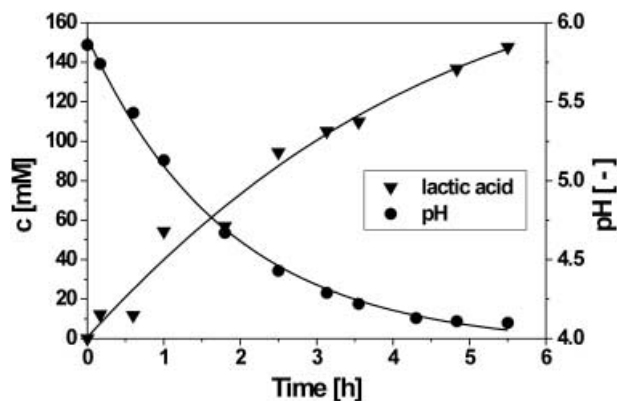


Fig. 2 Production of lactic acid (triangles) during a typical biotransformation of (2,5)-hexanedione to (2*R*,5*R*)-hexanediol and resulting decrease of pH (circles). Conditions: vol. =0.036 L, 30°C, pH 6, 0.5 M phosphate buffer, 462.5 mM glucose, 87 mM (2,5)-hexanedione, 6 g *L. kefir*

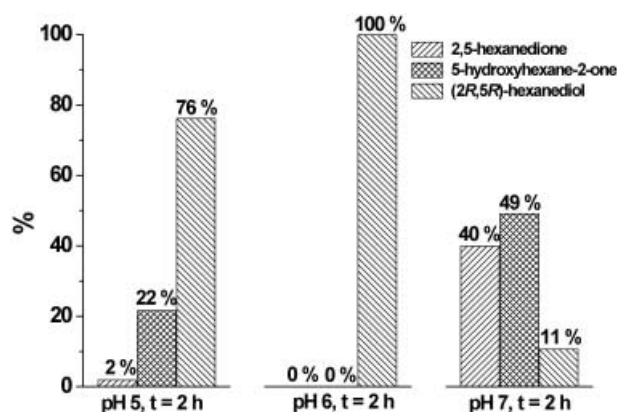


Fig. 3 Influence of pH on selectivity of the biotransformation after 2 h. Conditions: V=0.3 L, 30°C, 150 mM glucose, 100 mM (2,5)-hexanedione, 3 g *L. kefir*

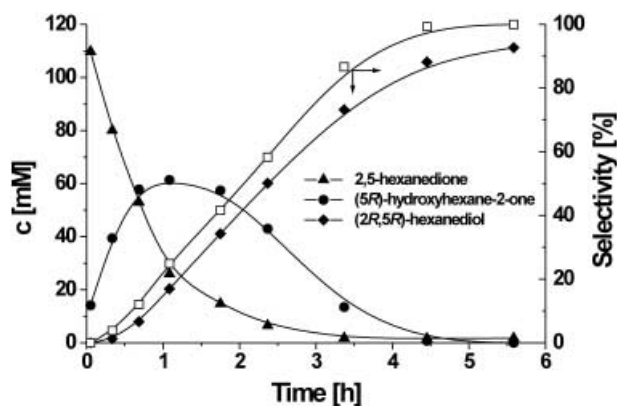


Fig. 4 Concentration (left axis) and selectivity (open squares; right axis) of a typical batch reaction as function of time. Conditions: V=0.03 L, 30°C, pH 6, 141 mM glucose, 110 mM (2,5)-hexanedione, 6 g *L. kefir*

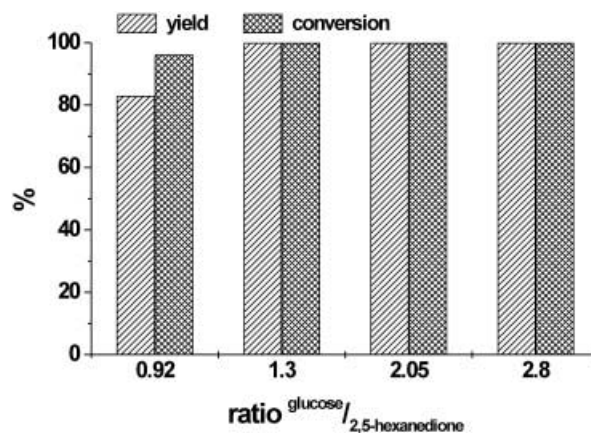


Fig. 5 Influence of different cosubstrate to substrate ratios on conversion and yield. Conditions: V=0.06 L, 30°C, pH 6, 6 g *L. kefir* ratio. 0.92 100 mM glucose, 109 mM (2,5)-hexanedione; ratio 1.3 141 mM glucose, 110 mM (2,5)-hexanedione; ratio 2.05 219 mM glucose, 107 mM (2,5)-hexanedione; ratio 2.8 290 mM glucose, 103 mM (2,5)-hexanedione

The course of a typical batch reaction is shown in Fig. 4. Because of the different reaction rates of the two subsequent reduction steps, an interim accumulation of the intermediate (5*R*)-hydroxyhexane-2-one is observed.

Ratio of cosubstrate to substrate

The optimum ratio of the cosubstrate, glucose, to the substrate, (2,5)-hexanedione, was determined. The final conversion and the final yield of (2*R*,5*R*)-hexanediol was investigated upon changing the initial ratio of both compounds. The ratios tested ranged from an excess of glucose (2.8) to a slight excess of the substrate (0.92). The results are shown in Fig. 5. Varying the ratio of glucose to (2,5)-hexanedione had no influence on the diastereo-selectivity. A ratio of 1:1 was chosen for further experiments.

Fed-batch with *L. kefir*

After determination of the minimum ratio of glucose to (2,5)-hexanedione, fed-batch experiments were performed. In this series of experiments, different feed rates for each compound were used. All fed-batch experiments were carried out in 500 mL shake flasks with a starting volume of 100 mL and cell suspensions of 10% wet cells.

The first set of experiments showed the influence of different feed rates. Because of the limited reaction volume, feed solutions were used at high concentration. The concentrations of the glucose and (2,5)-hexanedione solutions were both 2 M and the concentration of the NaOH solution used for pH titration was 4 N.

By reducing both feed rates from 24 mmol/h to 2.4 mmol/h, an increase of selectivity to 95% was

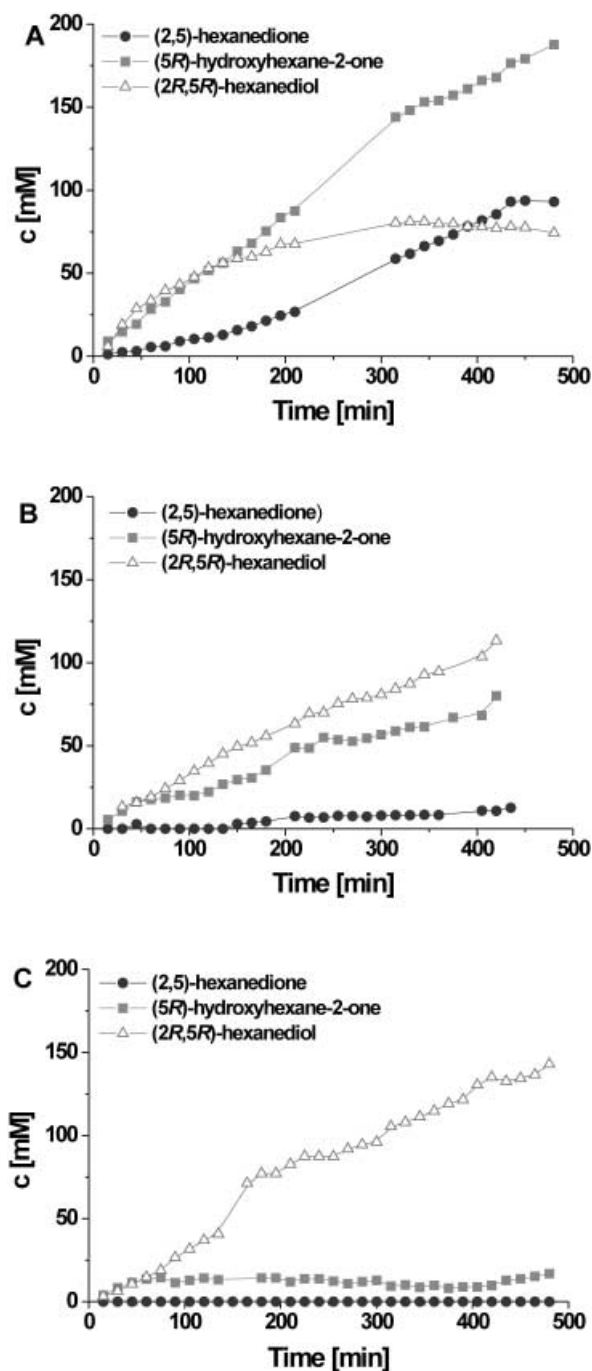


Fig. 6 Influence of different feed rates (A 24 mmol/h, B 7.2 mmol/h, C 2.4 mmol/h) of glucose and (2,5)-hexanedione on the course of the bioreduction. Conditions: $V_0 = 0.1$ L, 30°C, pH 6, glucose feed 2 M, (2,5)hexanedione feed 2 M, 10 g *L. kefir*. Feed rates (ml/h): A 12, B 3.6, C 1.2

achieved. The resulting concentration/time plots for one of these fed-batch series are shown in Fig. 6A–C.

Discussion

The bioreduction catalyzed by *L. kefir* was expected to be a very simple tool to produce enantiopure (*R,R*)-diols.

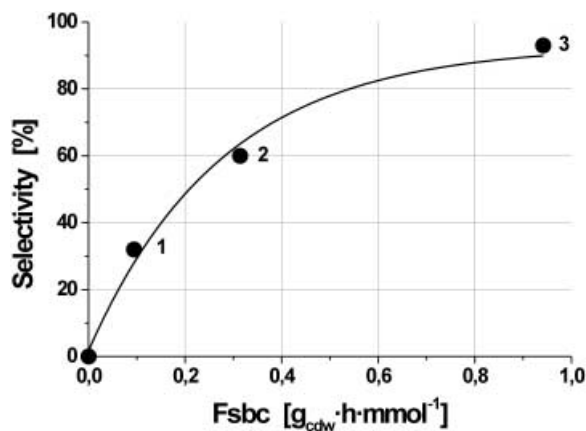


Fig. 7 Selectivity as a function of the feed specific biomass concentration ($fsbc$). Numbers show selectivities at points of highest conversion resulting from Fig. 6A–C: 1 A, 2 B, 3 C

By investigating the reaction conditions it could be shown that the selectivity towards the diol reached its maximum at a pH of 6. At this pH, a maximum reaction rate for the second reaction from the hydroxyketone to the diol was achieved. All subsequent experiments were carried out at pH 6 because the main product of interest was (2*R*,5*R*)-hexanediol.

In all cases with an excess of glucose over (2,5)-hexanedione, quantitative conversion and yield were reached. If less glucose was used (ratio 0.92), the reaction stopped at a conversion of 83% due to the lack of further reduction equivalents. In batch experiments, it could be shown that, per reduced oxofunction, 0.5 mol glucose was needed to achieve quantitative yield of (2*R*,5*R*)-hexanediol. In comparison to the production of (2*S*,5*S*)-hexanediol by *Saccharomyces cerevisiae* (Lieser 1983; Bel-Rhlid et al. 1992; Kometani et al. 1996), which needs a high excess of glucose, the reduction with *L. kefir* needs only equimolar amounts of substrate and cosubstrate.

By carrying out the fed-batch at pH 6 with a high feed rate of 24 mmol/h for (2,5)-hexanedione, enantiopure (5*R*)-hydroxyhexane-2-one could be synthesized (Fig. 6A). The accumulation of the dione was a result of the high feed rate because the total activity of the cell mass used was not high enough to convert all the starting material provided. By reducing the feed rates of (2,5)-hexanedione and glucose, the residual concentration of (2,5)-hexanedione is also reduced and conversion increased (Fig. 6B, C). Fig. 6A–C shows that with a decreasing feed rate of glucose/(2,5)-hexanedione at a constant ratio, selectivity is significantly increased.

For a generally applicable correlation between selectivity, feed rate and catalyst concentration the feed specific biomass concentration ($fsbc$) was defined:

$$fsbc = \frac{\text{biomass}}{\text{feed}} \doteq \frac{g \cdot h}{mmol} \quad (2)$$

Plotting the selectivity achieved at the end of the experiments shown in Fig. 6 as a function of the $fsbc$ (Fig. 7)

reveals that decreasing the substrate feed or increasing the catalyst concentration results in higher selectivity.

This information could be used for control of selectivity in fed-batch experiments. The parameters feed concentration and biomass concentration can be directly correlated to selectivity. This is an easy tool to optimize different fed-batch experiments towards quantitative selectivity of (2*R*,5*R*)-hexanediol.

Acknowledgements Part of this work was financially supported by the BMBF (project no. 0312639B) and Jülich Fine Chemicals (Jülich, Germany). The authors would like to thank Prof. C. Wandrey for his ongoing generous support.

References

- Ager DJ (1999) Handbook of chiral chemicals. Dekker, New York
- Akutagawa S (1995) Asymmetric synthesis by metal binap catalysts. *Appl Catal A*: 128:171–207
- Bel-Rhliid R, Fauve A, Renard MF, Veschambre H (1992) Microbiological reduction of carbonyl groupings: preparation of stereoisomeric acyclic chiral α -diols. *Biocatalysis* 6:319–337
- Bertau M (2001) Novel unusual microbial dehalogenation during enantioselective reduction of ethyl 4,4,4-trifluoro. *Tetrahedron Lett* 42:1267–1268
- Bertau M, Bürli M (2000) Enantioselective microbial reduction with baker's yeast on an industrial scale. *Chimia* 54:503–507
- Bradshaw CW, Hummel W, Wong C-H (1992) *Lactobacillus kefir* alcohol dehydrogenase: a useful catalyst for synthesis. *J Org Chem* 57:1532–1536
- Burk MJ, Feaster JE, Harlow RL (1991) New chiral phospholanes; synthesis, characterization, and use in asymmetric hydrogenation reactions. *Tetrahedron* 2:569–592
- Burk MJ, Gross MF, Martinez JP (1995) Asymmetric catalytic synthesis of β -branched amino acids via highly enantioselective hydrogenation. *J Am Chem Soc* 117:9375–9376
- Burk MJ, Gross MF, Harper TGP, Kalberg CS, Lee JR, Martinez JP (1996) Asymmetric catalytic routes to chiral building blocks of medicinal interest. *Pure Appl Chem* 68:37–44
- Caron G, Kazlauskas RJ (1994) Isolation of racemic 2,4-pentanediol and 2,5-hexanediol from commercial mixtures of racemic and meso sulfites. *Tetrahedron* 5:657–664
- Hummel W (1990) Reduction of acetophenone to R(+)-phenylethanol by a new alcohol dehydrogenase from *Lactobacillus*. *Appl Microbiol Biotechnol* 34:15–19
- Hummel W, Liese A, Wandrey C (1999) Process for reducing keto-group containing compounds. European Patent Application, EP 00113127.51–2110
- Ikeda H, Sako E, Sugai T, Ohta H (1996) Yeast-mediated synthesis of optically active diols with C2-symmetry and (R)-4-pentanolide. *Tetrahedron* 52:8113–8122
- Kandler P, Kunath O (1983) *Lactobacillus kefir* sp. nov, a component of the microflora of kefir. *Syst Appl Microbiol* 4:286–294
- Kometani T, Yoshii H, Matsuno R (1996) Large-scale production of chiral alcohols with bakers yeast. *J Mol Catal B* 1:45–52
- Liese A, Seelbach K, Buchholz A, Haberland J (2000) Processes. In: Liese A, Seelbach K, Wandrey C (eds) *Industrial biotransformations*. Wiley-VCH, Weinheim, pp 93–392
- Lieser JK (1983) A simple synthesis of (S,S)-2,5-hexandiol. *Synth Commun* 9:765–767
- Taylor SJC, Holt KE, Brown RC, Keene PA, Taylor IN (2000) Choice of biocatalyst in the development of industrial biotransformations. In: Patel RN (ed) *Stereoselective biocatalysis*. Dekker, New York, pp 397–404
- Wolberg M, Hummel W, Wandrey C, Müller M (2000) Highly regio- and enantioselective reduction of 3,5-dioxocarboxylates. *Angew Chem Int Ed Engl* 39:4306–4308