

A Continuously Operated Bimembrane Reactor Process for the Biocatalytic Production of (2*R*,5*R*)-Hexanediol

Kirsten Schroer[†] and Stephan Lütz^{*,†}

Institute of Biotechnology 2, Research Centre Jülich, 52425 Jülich, Germany

Abstract:

Alcohol dehydrogenase-catalyzed reductions of prochiral ketones to chiral alcohols require the regeneration of consumed cofactors such as NADH or NADPH. In the substrate-coupled cofactor regeneration approach, where 2-propanol is oxidized to acetone, complete conversion is inhibited by a thermodynamic limitation. This can be overcome by applying methods of *in situ* product removal techniques such as pervaporation. Here we present a new reactor concept which enables a continuous biocatalytic ketone reduction process with concurrent *in situ* removal of the byproduct acetone. In such a bimembrane reactor system recombinant *Escherichia coli* cells expressing alcohol dehydrogenase from *Lactobacillus brevis* were applied for the continuous reduction of 2,5-hexanedione. The product (2*R*,5*R*)-hexanediol could be synthesized with exceedingly high space-time yield of >170 g/(L·d) and catalyst usage (17.9 g_p/g_{wetcellweight}).

Introduction

Reduction of prochiral ketones to optically active compounds by biocatalytic methods is of particular interest due to the high regio-, stereo-, and enantioselectivity of biocatalysts.¹ This type of reaction is usually catalyzed by alcohol dehydrogenases (ADH) which are dependent on nicotinamide dinucleotide cofactors such as NADH or NADPH.² In contrast to enzyme-catalyzed processes, where NAD(P)H must be added, whole-cell catalysts already deliver a certain amount of intracellular cofactors. The reduction of 2,5-hexanedione to optically pure (2*R*,5*R*)-hexanediol is of particular interest since (2*R*,5*R*)-hexanediol is a versatile building block for the synthesis of various chiral phosphine ligands, which are used in chiral Wilkinson catalysts.^{3–5}

To run a NAD(P)H-dependent process economically efficiently there is a need for cofactor-regenerating reactions which reduce NAD(P)⁺ to NAD(P)H by oxidizing a cosubstrate.^{6,7} In the literature different methods for cofactor regeneration are

described. One of them is the substrate-coupled approach, where the producing ADH also catalyzes the cofactor-regenerating reaction. Usually 2-propanol is applied as cosubstrate for that purpose.

The major disadvantage of substrate-coupled cofactor regeneration is the thermodynamic limitation that occurs since there is an equilibrium between all involved educts and products. The thermodynamic equilibrium can be shifted towards higher product yields either by increasing the concentration of substrate and/or cosubstrate or by removing the product or the coproduct acetone from the reaction process.⁸

Results and Discussion

The production of (2*R*,5*R*)-hexanediol starting from the diketone 2,5-hexanedione in combination with substrate-coupled cofactor regeneration is shown in Figure 1. For every molecule of 2,5-hexanedione that should be converted to (2*R*,5*R*)-hexanediol two molecules of NAD(P)H are needed, and thus at least a 2-fold excess of the cosubstrate 2-propanol related to the substrate 2,5-hexanedione is also needed. However, as pointed out in Figure 2, a much higher excess of 2-propanol is necessary to achieve sufficient yield since remarkable amounts of the intermediate 5-hydroxyhexane-2-one are produced which are not converted to the product 2,5-hexanediol. From the results presented in Figure 2 an equilibrium constant of $k_{eq} \approx 0.1$ can be calculated. Thus, this reaction system is a particularly good example to demonstrate the impact of acetone removal due to its thermodynamic properties.

There are already a couple of methods described in the literature dealing with *in situ* removal of products or byproducts during biocatalytic processes. *In situ* acetone removal has already been applied for enzyme-catalyzed processes⁹ and also for whole-cell processes.^{10,11} In all cases significantly higher yields could be achieved compared to yields from processes without removal of the byproduct acetone from the biotransformation process, but this strategy has only been applied to biotransformation processes operated in batch mode.

Whole cells are particularly suitable catalysts to be applied in continuously operated processes.¹² Due to the natural compartmentation achieved by the cell membrane, there is a high retention of cofactors within the cytosol, and thus such a

* Corresponding author. Telephone: (+49)(0)2461-61-4388. Fax: (+49)(0)2461-61-3870. E-mail: s.luetz@fz-juelich.de.

[†] Present address: Novartis Institutes for Biomedical Research, 4056 Basel, Switzerland.

- (1) Goldberg, K.; Schroer, K.; Lütz, S.; Liese, A. *Appl. Biotechnol. Microbiol.* **2007**, *76*, 237–248.
- (2) Breuer, M.; Ditrach, K.; Habicher, T.; Hauer, B.; Kessler, M.; Stürmer, R.; Zelinski, T. *Angew. Chem., Int. Ed.* **2004**, *43*.
- (3) Burk, M. J.; Feaster, J. E.; Harlow, R. L. *Organometallics* **1990**, *9*, 2653–2655.
- (4) Burk, M. J. *J. Am. Chem. Soc.* **1991**, *113*, 8518–8519.
- (5) Burk, M. J.; Feaster, J. E.; Harlow, R. L. *Tetrahedron: Asymmetry* **1991**, *2*, 569–592.
- (6) Wandrey, C. *Chem. Rec.* **2004**, *4*, 254–265.
- (7) Wichmann, R.; Vasic-Racki, D. *Adv. Biochem. Eng. Biotechnol.* **2005**, *92*, 225–260.

- (8) Lye, G. J.; Woodley, J. M. *Trends Biotechnol.* **1999**, *17*, 395–402.
- (9) Stiller, T.; Bönitz, M.; Villela, M.; Liese, A. *Chem. Eng. Technol.* **2002**, *74*, 1035–1039.
- (10) Goldberg, K.; Edegger, K.; Kroutil, W.; Liese, A. *Biotechnol. Bioeng.* **2006**, *95*, 192–198.
- (11) Schroer, K.; Tacha, E.; Lütz, S. *Org. Process Res. Dev.* **2007**, *11*, 836–841.
- (12) Rao, N. N.; Lütz, S.; Würges, K.; Minör, D. *Org. Process Res. Dev.* **2009**, *13*, 607–616.

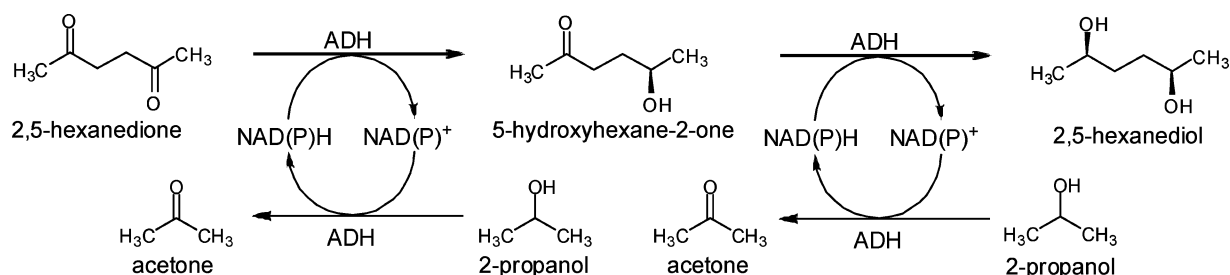


Figure 1. Biocatalytic reduction of 2,5-hexanedione with substrate-coupled cofactor regeneration.

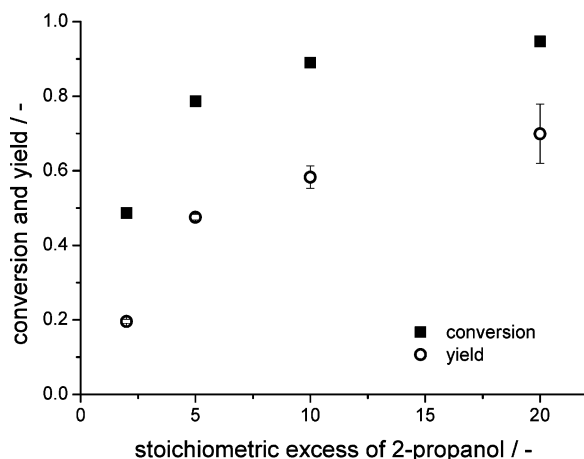


Figure 2. Correlation between conversion, yield, and the stoichiometric excess of 2-propanol. Yield is defined as amount of product (2*R*,5*R*)-hexanediol divided through the initial amount of substrate. Conversion is defined as amount of substrate converted to intermediate or product divided through the initial amount of substrate.

process can be carried out over a long time period without addition of external cofactors.¹³ Haberland et al. already reported the continuous production of (2*R*,5*R*)-hexanediol by whole-cell catalysis.¹⁴ Wild-type cells of *Lactobacillus kefir* DSM 20587 were applied as biocatalysts, and cofactor regeneration was achieved by addition of glucose. During the continuously operated biotransformation process a space-time yield of 64 g·L⁻¹·d⁻¹ and a catalyst usage of 15 g_{product}/g_{biomass} could be achieved. However, due to the low stability of the biocatalyst the process could only be operated over a time period of 5 days.

In previous studies we developed a whole-cell biotransformation setup where the byproduct acetone was removed by pervaporation. There the reduction of 2,5-hexanedione was carried out in a batch reaction.¹¹ Here we now present an enhanced reactor concept which combines an *in situ* acetone removal technique with a continuously operated biotransformation process. This concept offers the possibility to remove the coproduct acetone from a continuously operated biotransformation process when cofactor regeneration is carried out in the substrate-coupled approach by applying 2-propanol as cosubstrate. Thus, thermodynamically unfavorable reactions can be catalyzed continuously. Figure 3 shows the reactor setup including two membrane modules: an ultrafiltration membrane

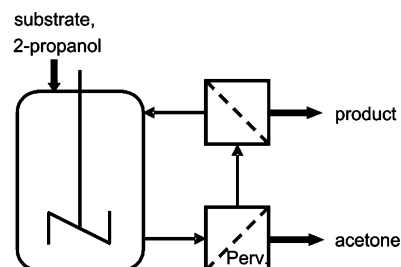


Figure 3. Reactor setup for *in situ* acetone removal by pervaporation during continuously operated biotransformation processes with cell retention.

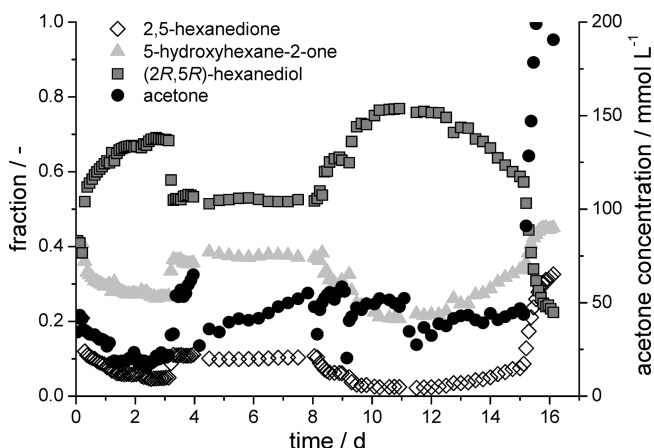


Figure 4. Fraction of 2,5-hexanedione, 5-hydroxyhexane-2-one, (2*R*,5*R*)-hexanediol, and acetone concentration during a continuously operated biotransformation process with *in situ* acetone removal by pervaporation. Process conditions: see Table 2.

for retention of the whole-cell biocatalyst and a pervaporation membrane for *in situ* acetone removal.

Recombinant *Escherichia coli* cells expressing alcohol dehydrogenase from *L. brevis* (LbADH)¹⁵ were applied as biocatalyst. In former studies this whole-cell biocatalyst turned out to be highly effective and robust especially at elevated concentrations of substrates and 2-propanol.¹³

In the continuously operated biotransformation process with *in situ* acetone removal different concentrations of the substrate 2,5-hexanedione and the cosubstrate 2-propanol were applied (see Table 2, Experimental Section). Figure 4 shows the fraction of the substrate 2,5-hexanedione, the intermediate 5-hydroxyhexane-2-one, and the product (2*R*,5*R*)-hexanediol during the biotransformation process. Furthermore the acetone concentration is displayed. Due to the *in situ* acetone removal technique the acetone concentration did not exceed more than 50

(13) Schroer, K.; Mackfeld, U.; Tan, A. W. I.; Wandrey, C.; Heuser, F.; Bringer-Meyer, S.; Weckbecker, A.; Hummel, W.; Daussmann, T.; Pfaller, R.; Liese, A.; Lütz, S. *J. Biotechnol.* **2007**, *132*, 438–444.

(14) Haberland, J.; Hummel, W.; Daussmann, T.; Liese, A. *Org. Process Res. Dev.* **2002**, *6*, 458–462.

(15) Hummel, W. *Adv. Biochem. Eng. Biotechnol.* **1997**, *58*, 145–184.

Table 1. Process parameters

space-time yield	172 g·L ⁻¹ ·d ⁻¹
maximum yield	77%
overall amount of product	287 g
enantiomeric excess, diastereomeric excess	>99% ^a
catalyst usage	17.9 g _P /g _{wet cell weight}
deactivation constant k_{des}	0.061 d ⁻¹

^a Determined for purified product.

mmol·L⁻¹. This corresponds to 90% of acetone removal. This value was calculated for the time period between 10 and 13 days, where the highest yield of (2*R*,5*R*)-hexanediol was achieved. The resulting product concentration was about 225 mmol·L⁻¹. During that time period a 5-fold excess of 2-propanol and a residence time of 4 h were applied. According to Figure 3 and the derived equilibrium constant k_{eq} a process without acetone removal would result in ~60% yield under these conditions. After switching off the vacuum at day 15, the conversion dramatically dropped down. This effect also demonstrates that such a high yield of (2*R*,5*R*)-hexanediol can only be achieved when acetone is removed from the biotransformation process.

Table 1 shows important process parameters achieved during the continuously operated biotransformation process with *in situ* acetone removal by pervaporation. The maximal space-time yield of 172 g·L⁻¹·d⁻¹ is remarkable since it is a 3-fold increase compared to that by Haberland et al.¹³ Moreover, the process could be carried out for 15 instead of 5 days. The deactivation constant k_{des} was estimated from the linear decrement of conversion during the time period of $t = 12$ days and $t = 15$ days of the continuously operated biotransformation process.

During the process a small concentration of 0.01 mmol·L⁻¹ NADP was fed into the reaction system constantly. In principle there is no need for addition of cofactors to a whole-cell biotransformation process. The applied biocatalyst was already used in former whole-cell biotransformation processes. Even at elevated 2-propanol concentrations the recombinant *E. coli* cells expressing *LbADH* turned out to show a high operational stability with excellent membrane integrity and thus a retention of intracellular cofactors.¹² But here higher yields were obtained with the addition of cofactors in comparison to processes without addition of cofactors, which was tried in a first biotransformation process (data not shown). In order to demonstrate the benefit of acetone removal techniques for continuously operated biotransformation processes we did not focus on optimization of reaction conditions with respect to membrane integrity and cofactor retention. By optimizing the process parameters, in particular substrate and cosubstrate concentration, it might be possible to run such a process without addition of cofactors with comparable or even better process parameters. The presented reactor concept offers further possibilities for optimization, e.g. more efficient acetone removal by applying pervaporation membranes with better selectivity for acetone and by applying higher membrane areas. The process presented here was carried out at 40 °C reaction temperature in order to enable a high acetone removal rate. By providing a higher membrane area and a higher membrane flux of acetone it might be possible to lower the reaction temperature which would have a positive effect on the stability of the biocatalyst.

Table 2. Process conditions

time period [days]	2,5-hexanedione [mmol·L ⁻¹]	2-propanol [mmol·L ⁻¹]	residence time τ [h]	wet cell weight [g·L ⁻¹]
0–1	100	500	2	100
1–2			2.67	
2–3				200
3–4	300	1000		
4–8			4	
8–9		1500		
9–16		3000		

Experimental Section

Recombinant *E. coli* BL21 Star (DE3) cells carrying the plasmid pBtac-*lbadh* (X-zyeme, Düsseldorf, Germany) and thus overexpressing alcohol dehydrogenase from *L. brevis* were applied as biocatalysts. Cells were cultivated in modified Luria–Bertani (LB) medium: 10 g·L⁻¹ casein peptone, 5 g·L⁻¹ yeast extract, 10 g·L⁻¹ NaCl, 4 g·L⁻¹ glucose. Fifty milliliters of medium containing 50 mg·L⁻¹ ampicillin were inoculated with 0.5 mL of a glycerol stock culture. After incubation overnight at 30 °C and 150 rpm, 200 mL of the same medium was inoculated with 0.5 mL of the preculture. Main cultures were incubated for 6 h at 37 °C and 150 rpm. Then gene expression was induced by adding 0.2 mmol·L⁻¹ IPTG. Afterwards the cultures were incubated for 18 h at 27 °C and 150 rpm. The cells were harvested by centrifugation (Beckman Coulter Avanti J-20 XP, 8000 rpm, 20 min, 4 °C) and washed once with 0.05 mol·L⁻¹ potassium phosphate buffer (pH 6.0).

The biotransformation process was carried out in 0.05 mol·L⁻¹ potassium phosphate buffer (pH 6.0) with varying concentrations of 2,5-hexanedione, 2-propanol and biomass and varying residence time (see Table 2) at 40 °C. The NADP concentration was 0.01 mmol·L⁻¹ during the whole process, and the vacuum was kept constant at 100 mbar by a diaphragm vacuum pump (MZ 2C, Vacuubrand, Wertheim, Germany) The vacuum was stopped after 15 days of process time.

The reactor setup consisted of a ultrafiltration membrane, equipped with a 10 kDa cutoff Omega-membrane (Pall, New York, U.S.A.), a pervaporation membrane module (Mechanical Workshop of the Research Centre Juelich, Jülich, Germany), and a peristaltic pump (505U, Watson-Marlow, Rommerskirchen, Germany) which ensured adequate circulation of the biotransformation mixture through the tube system and the associated membrane modules. The polymethoxysiloxane membrane PA-HP-02 (PolyAn, Berlin, Germany) with a membrane area of 176 cm² was applied as the pervaporation membrane. The overall reaction volume was 80 mL.

A 200 μ L sample of reaction solution was removed from the reaction system and centrifuged (centrifuge 5415D, Eppendorf, Hamburg, Germany) at 16000 rpm for 30 s to remove the cells. To avoid further reaction catalyzed by leaked enzyme, the supernatant was transferred into a second tube and incubated for 60 s at 99 °C (ThermoStat plus, Eppendorf, Hamburg, Germany). After cooling at 4 °C the sample was prepared for gas chromatography analysis.

Quantification of all substrates and products was carried out on an Agilent HP-6890 A gas chromatograph with a Permabond Carbowax 20 M column (50 m $\times$ 0.32 mm i.d., Macherey-Nagel, Düren, Germany) with a flame ionization detector and

helium as carrier gas. To minimize the injection error, *n*-butanol was used as internal standard. The following temperature program was used: 5 min 50 °C, 40 °C min⁻¹, 9 min 160 °C. Typical retention times were acetone 2.5 min, 2-propanol 4.0 min, *n*-butanol 7.1 min, 2,5-hexanedione 10.3 min, 5-hydroxyhexane-2-one 11.7 min, 2,5-hexandiol 15.6 min. All substances were quantified on the basis of their respective response factors with standard dilutions.

The determination of equilibrium constants was carried out by incubation of 50 g·L⁻¹ recombinant *E. coli* in 0.05 mol·L⁻¹ potassium phosphate buffer (pH 6.0) with 0.1 mol·L⁻¹ 2,5-hexanedione and varying concentrations of 2-propanol at room temperature on 1 mL scale in Eppendorf tubes. After 24 h samples were taken and analyzed by GC as described above.

For isolation and purification of (2*R*,5*R*)-hexanediol the product solution was extracted with ethyl acetate. The solvent was removed from the resulting organic phase by vacuum evaporation (Rotavapour R-114 and vacuum pump Vac V-513, Büchi Labortechnik, Konstanz, Germany) at 30 mbar and 40 °C. The remaining compounds were separated by column chromatography with silica gel as stationary phase and ethyl acetate as mobile phase.

Acknowledgment

We thank U. Mackfeld for technical assistance and their cooperation partners Dr. Bringer-Meyer (IBT-1, Research Centre Juelich), Dr. W. Hummel (IMET, Heinrich-Heine University Düsseldorf), Dr. J. Pietruszka (IBOG, Heinrich-Heine University Düsseldorf), Dr. Dausmann and Dr. Hennemann (Codexis), Dr. Pfaller, Dr. Stohrer and Dr. Wich (Wacker Chemie). Furthermore, we thank PolyAn for supplying the pervaporation membrane. This work was financially supported by BMBF (Project no. 0313440C).

Supporting Information Available

Information on the mass flow and selectivities of the pervaporation membrane as well as the 2-propanol concentrations during the biotransformation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Received for review July 2, 2009.

OP9001643