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New Continuous Production Process for Enantiopure (2*R*,5*R*)-Hexanediol

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Abstract:

A new continuous production process has been developed for optically active pure (2*R*,5*R*)-hexanediol. The process uses resting whole cells of *Lactobacillus kefir* DSM 20587 as a biocatalyst. The reduction of (2,5)-hexanedione to (2*R*,5*R*)-hexanediol was carried out in a 2-L continuously operated membrane reactor. Conversion of (2,5)-hexanedione was nearly quantitative and the selectivity between product and intermediate was 78% for the product. Enantioselectivity and diastereoselectivity were >99% for the whole period. The productivity of *L. kefir* could be increased by factor 30. (2*R*,5*R*)-Hexanediol was continuously produced over 5 days with a space−time yield of 64 g·L^{-1}·d^{-1}.

Introduction

Enantio- and diastereomerically pure diols are important and interesting building blocks for the synthesis of pharmaceuticals, agrochemicals, and fine chemicals.[1] (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.[2,3,4]

Here we present the application of resting whole cells from *Lactobacillus kefir* DSM 20587 as a biocatalyst[5,6] for the enantio- and diastereoselective reduction of (2,5)-hexanedione **1** to (2*R*,5*R*)-hexanediol **3**. The reaction is shown in Figure 1. The respective enantio- and diastereoselectivities of the product were >99%.

In the literature there are several chemical routes published leading to (2*R*,5*R*)-hexanediol. In principle only three different approaches are used. The first is the use of compounds from the chiral pool to introduce chirality. D-Mannitol is a possible starting compound. In a three-step reaction sequence **3** can be synthesized in high optical purities, but the overall yield is only 30%.[7] The second approach is the use of oxazaborolidines in tetrahydrofurane as the solvent. At 75% the described yields are moderate and only 45% ee was reached.[8,9] The third approach uses ruthenium−BINAP complexes as catalysts for the reduction. The optical purities seem to be moderate, but the yields are rather low 15−50%.[10]

Biotechnological approaches are not often found in the literature, although they are used for production purposes. The established industrial production process for (2*R*,5*R*)-hexanediol is a multistep synthesis starting with an enantioselective acylation of the (*R*)-hydroxy function of the racemic/meso (2,5)-hexanediol mixture catalyzed by a lipase.[11,12] Subsequently, the nonacylated (*S*)-hydroxy function of the meso-(*R*,*S*)-diol is inverted by chemical transformation with methane sulfonyl chloride leading to the (*R*,*R*)-diol. Reagents used in the production process are triethylamine, methane sulfonyl chloride, dichloromethane, dimethyl formamide, cesium acetate, methanol, and acidic resins (Amberlite IR 120). The maximum theoretical yield of this process is 75%. However, no information has been published on the real yields achieved in this process. Another microbial approach using resting whole cells to produce (2*R*,5*R*)-hexanediol was published by Ohta et al. in 1996.[13] *Pichia farinosa* is used as a biocatalyst to reduce (2,5)-hexanedione to (2*R*,5*R*)-hexanediol. In batch experiments yields of about 83% are achieved. The enantiomeric excess is >99%, but the diastereomeric excess is only >95%. The productivity is very low in this process (12.5 $mg_{product}/g_{wetweight}$).

The production process introduced in this contribution starts from a much cheaper substrate than that used industrially, and in comparison to the whole-cell approach described by Ohta et al. (see Continuous Reduction section) higher productivities were achieved. Furthermore, the reduction with *L. kefir* leads to quantitative optical purities. After extraction with ethyl acetate and crystallization from isooctane the product is isolated in its optically pure form. A further

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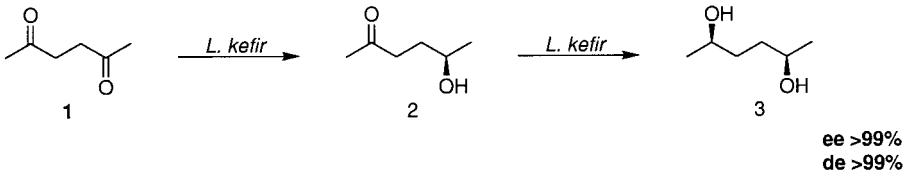

Figure 1. **Microbial reduction of (2,5)-hexanedione to (2*R*,5*R*)-hexanediol with *Lactobacillus kefir* DSM 20587.**

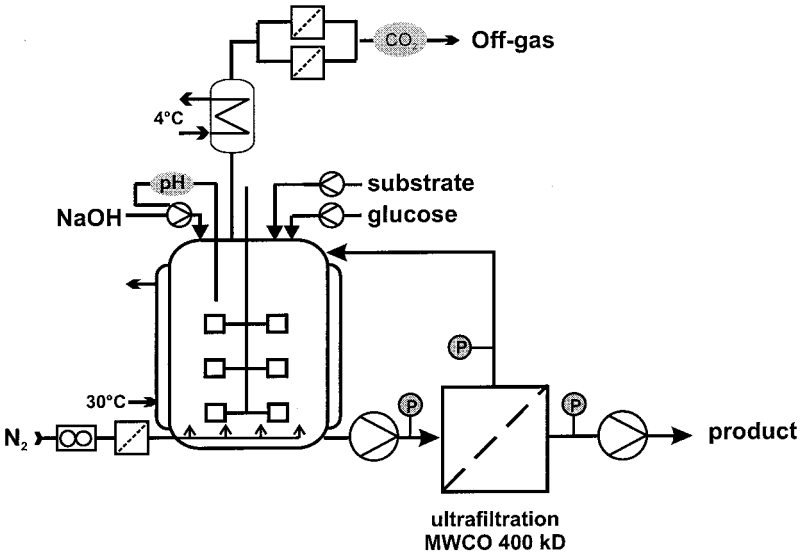

Figure 2. **Flow scheme of the continuously operated stirred tank reactor with cell retention.**

advantage is the realization of a continuous production process.

The metabolism of the cell is used to regenerate the cofactor NADPH by providing glucose as cosubstrate. This is another advantage of the whole cell approach because the in vitro regeneration of NADP to NADPH is not easy to realize. Glucose is metabolized to lactate, acetate, ethanol, and carbon dioxide.

To our knowledge this is the first continuously operated process using *L. kefir* as a biocatalyst for the synthesis of optically pure diols.

Reactor Setup

Cells of *L. kefir* were cultivated, harvested, and stored at temperatures −20 °C. Thus, the amount of cells used in each experiment is easy to prepare (see Experimental Section).

The continuous reductions were carried out on a 2-L scale under anaerobic conditions. For cell retention an ultrafiltration module with a membrane area of 1.7 m^2 and a molecular weight cutoff of 400 kD was used. A buffered glucose solution was the main feed to the reactor. The substrate itself was provided in a pure form at a very low flow rate. This setup enabled easy variation of the substrate-to-glucose ratio. Variations of the substrate concentration and also a change to a different starting material can be realized. The reactor setup is shown in Figure 2. The residence time in the reactor was kept constant at 4 h for all experiments.

The pH was kept constant during biocatalysis at a value of 6 by adding 4 N sodium hydroxide solution. This addition was necessary, because of the large quantities of lactate and acetate produced by the cells. The temperature of the fermenter was 30 °C, the condenser temperature was 4 °C. Nitrogen gas was applied at 3 vvm to maintain anaerobic conditions and to increase the gas flow of carbon dioxide for off-gas analysis.

Continuous Reductions

The reduction of (2,5)-hexanedione proceeds in a two-step reaction. In the course of the reaction first the hydroxyketone **2** is formed and, depending on the reaction conditions, accumulated. Subsequently, this intermediate is further reduced to the diol.[6] Since the first reduction step is faster than the second one there is always an accumulation of the intermediate. Therefore the selectivity between **2** and **3** is an important parameter for product purity and can be defined as follows:

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

A biomass concentration of about 100 $g \cdot L^{-1}$ wet cells (approximately 21 $g \cdot L^{-1}$ cell dry weight (cdw)) was applied in the continuous reduction. The feed rates were 76 $mmol \cdot h^{-1}$ for glucose and 56 $mmol \cdot h^{-1}$ for (2,5)-hexanedione. The pH was set to 6 and kept constant by feeding sodium hydroxide solution controlled by a pH-stat. The residence time was set to 4 h resulting in an overall flux of 500 $mL \cdot h^{-1}$. The resulting concentration time course for **1**, **2**, and **3** is shown in Figure 3.

In the steady-state, a concentration of 90 $mmol \cdot L^{-1}$ of (2*R*,5*R*)-hexanediol **3** was established. The optical purity of the product was very high (de, ee > 99%). As seen in Figure 1, the concentration for the intermediate **2** was 30 $mmol \cdot L^{-1}$ and for **1** around 2 $mmol \cdot L^{-1}$. A space−time yield of 63.8

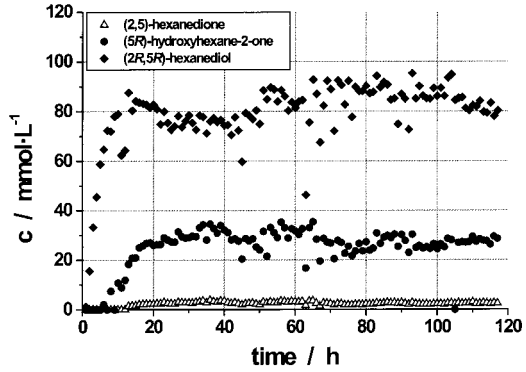

Figure 3. **Concentration time course for the continuous production of (2*R*,5*R*)-hexanediol with *L. kefir*. Conditions: $V = 2$ L, 30 °C, pH 6, $\tau = 4$ h, 0.05 mol·L^{-1} phosphate buffer, $c_{glucose}$ 152 mmol·L^{-1}, $c_{(2,5)-hexanedione}$ 112 mmol·L^{-1}, 21 g_{cdw} *L. kefir*.**

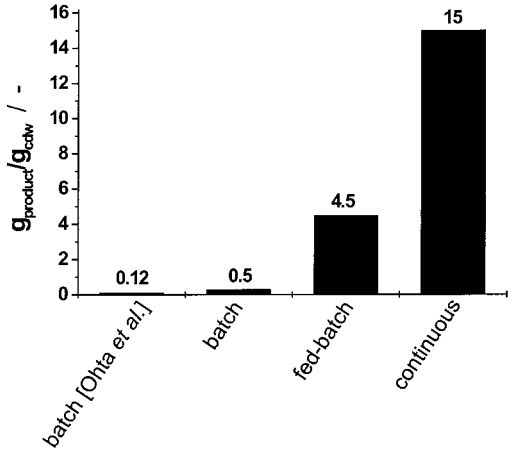

Figure 4. **Increase of product yield per g_{cdw} used per experiment.**

g·L^{-1}·d^{-1} could be achieved. The productivity of the cells was increased drastically in comparison to previously used production methods such as batch or fed-batch.[6] Because biomass is the cost-limiting factor the improvement of the ratio of $g_{product}/g_{cdw}$ is the key step towards a low-priced enantiopure product. This ratio was increased by factor 30 (Figure 4).

The conversion is nearly quantitative over a range of 120 h. A selectivity of 78% for (2*R*,5*R*)-hexanediol was achieved. In Figure 5 the conversion and the selectivity is plotted as function of time. Selectivity is an important parameter for downstream processing because crystallization of **3** is not possible if the selectivity is lower than 60%.

By changing the feed rate of (2,5)-hexanedione during continuous reduction different steady states of the reactor are achieved. In Figure 6 the concentration time course of **1**, **2**, and **3** for two different steady states is shown. The feed ratio of glucose to (2,5)-hexanedione was changed between the steady states (0−54 h: glucose feed 75 mmol·h^{-1}, (2,5)-hexanedione feed 38 mmol·h^{-1}; 55−95 h: glucose feed 75 mmol·h^{-1}, (2,5)-hexanedione feed 60 mmol·h^{-1}). All other conditions were described in the Reactor Setup section.

In Figure 7 it can be seen that the reproducibility between different continuous experiments is very high. The selectivity for diol formation increases with increasing conversion.

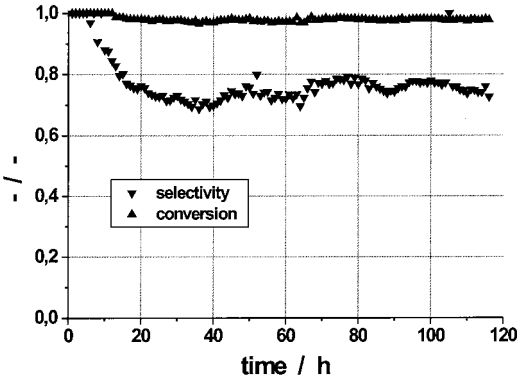

Figure 5. **Conversion and selectivity as function of time. Conditions: see Figure 3.**

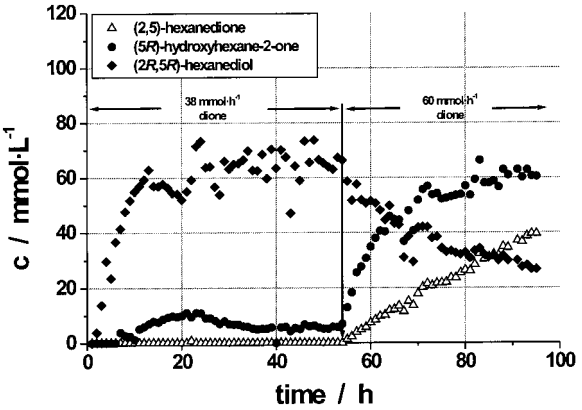

Figure 6. **Concentration time course for different steady states during the reduction of (2,5)-hexanedione. Conditions: $V = 2$ L, 30 °C, pH 6, $\tau = 4$ h, 0.05 mol·L^{-1} phosphate buffer, $c_{glucose}$ 152 mmol·L^{-1}, $c_{(2,5)-hexanedione}$ 76 mmol·L^{-1} (0−54 h), $c_{(2,5)-hexanedione}$ 120 mmol·L^{-1} (55−95 h), 21 g_{cdw} *L. kefir*.**

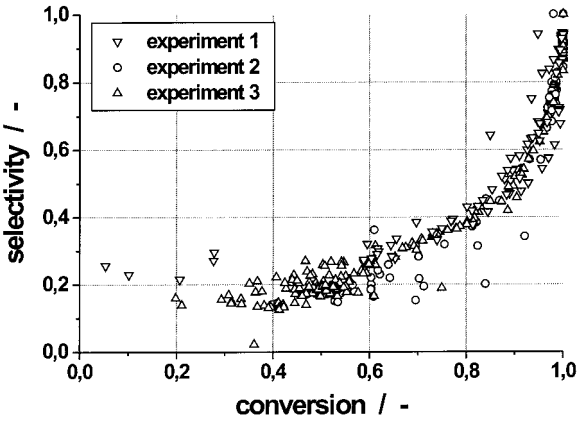

Figure 7. **Selectivity (eq 1) as function of conversion for different continuous reduction processes.**

Additionally, it is important to note that almost quantitative conversion is needed to reach the high selectivity necessary for easy downstream processing.

Feed-Specific Biomass Concentration

Beside the target molecule, (2*R*,5*R*)-hexanediol **3**, also the intermediate, (5*R*)-hydroxyhexane-2-one **2**, is of great industrial interest. One stereogenic center with an ee of >99% and a second prochiral functionality that offers a broad

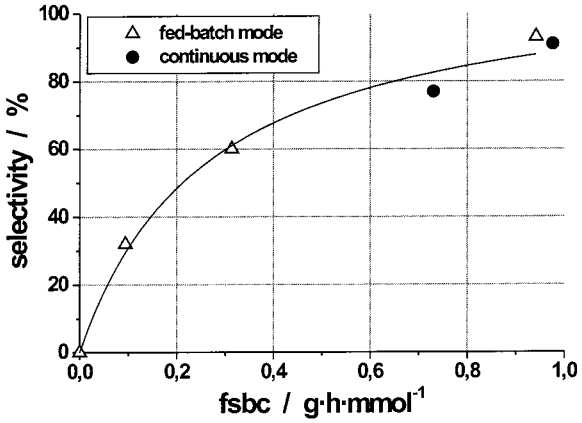

Figure 8. **Selectivity as function of feed-specific biomass concentration (fsbc) for fed-batch (triangles) and continuous mode (circles).**

spectrum of possible reactions creates an interesting building block.

The two parameters influencing the selectivity for the synthesis of (2*R*,5*R*)-hexanediol **3** are the amount of biomass and the feed rate of the substrate. The outcome of this is the definition of the feed-specific biomass concentration (fsbc), which correlates both parameters to the selectivity of the following reaction.

$$\text{fsbc} = \frac{\text{biomass}}{\text{feedrate}} \qquad (2)$$

We have already described the use of the fsbc[6] for controlling selectivity in the fed-batch mode. This can be seen in Figure 8 (triangles). Calculating the fsbc for the continuous steady states, it can be shown that these data points match the curve (Figure 8 (circles)). This means that it is possible to control the selectivity by varying these two parameters.

Therefore it should be possible to choose these two parameters in such a way as to produce only the intermediate, (5*R*)-hydroxyhexane-2-one **2**, not only in a fed-batch mode but also in a continuous mode.

Conclusions

A new continuous process for the diastereomeric production of (2*R*,5*R*)-hexanediol (ee >99%, de>99%) has been developed. By changing from batch to a continuously operated process the ratio of $g_{product}/g_{cdw}$ was increased by factor 30 to 15 g/g. A typical space-time yield for the continuous process over 5 days is 64 $g \cdot L^{-1} \cdot d^{-1}$.

Experimental Section

Biomass Production. *L. kefir* 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 hydrogen citrate, 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 cultivation at 30 °C on a reciprocating shaker set to 60 rpm five 100-mL cultures were added to a 10-L glass fermenter containing 9.5 L of the above-mentioned medium. The fermenter was stirred at 100 rpm for 2 days at a temperature of 30 °C. After finishing this intermediate culture the whole suspension was transferred into a 300 L fermenter (Chemap AG, Volketswil, Switzerland) containing 190 L of MRS medium. After a further 2 days of cultivation at 30 °C (300 rpm), $OD_{660\ nm} = 4.5$ was reached, and the cells were harvested with a cell separator (Typ SA 1, Westfalia Separator AG, Oelde, Germany) and stored at −20 °C. 1.2 kg of wet cells was obtained.

Continuous Reduction Procedure. All biotransformations were carried out anaerobically in a 3-L Labfors bioreactor with a 2-L working volume ($H = 38$ cm, $D = 11.6$ cm, Infors, Basel, Switzerland). The reactor was sterilized (121 °C for 20 min) prior to use, glucose solution was sterilized by filtration in a presterilized vessel (autoclavable sterile capsule 0.45- and 0.2-mm pores, Sartorius AG, Goettingen, Germany). The stirrer speed was 1200 rpm, the fermenter temperature was set at 30 °C, and the condenser temperature at 4 °C, controlled by a water bath and cryostat. The applied nitrogen flow was 6 $L \cdot min^{-1}$ (6 vvm). The pH was kept at 6 ± 0.05 with 4 M NaOH via a pH-stat (Metrohm, Herisau, Switzerland). The carbon dioxide concentration was measured in the off-gas (Fisher-Rosemount carbon dioxide analyzer type Binos 100 range 0−2%). LABVIEW 6.01 software (National Instruments, Munich, Germany) was used for online data acquisition. For cell retention an ultrafiltration module (KrosFlow, ϕ fibers 0.5 mm, A 1.7 m^2, MWCO 400 kD, Membrapure, Bodenheim, Germany) was integrated in an external loop (dead volume of loop approximately 200 mL, flux 60 $L \cdot h^{-1}$, permeate flux 500 $mL \cdot h^{-1}$). The filtration module was sterilized before and after operating the reactor by rinsing with 1 M NaOH solution. Cells (220 g) were thawed and washed twice with phosphate buffer (pH 6) and transferred to the fermenter. Antifoam (0.2 mL)was added. Nitrogen flow was started, and the cells were stirred for 15 min to remove all the carbon dioxide from the suspension. After 15 min the filtration and both feeds were started. Glucose was fed with 76 $mmol \cdot h^{-1}$ and a flow of 460 $mL \cdot h^{-1}$. (2,5)-Hexanedione was fed pure with 56 $mmol \cdot h^{-1}$ and a flow of 7 $mL \cdot h^{-1}$.

Product Purification. From the gathered permeate solutions the water was removed by reduced pressure (rotary evaporator Rotavapor R111, Büchi Laboratoriumstechnik GmbH, Göttingen, Germany). The concentrated aqueous solution (500 mL) was extracted five times with ethyl acetate (500 mL). Ethyl acetate was evaporated from the combined organic phases. Subsequent addition of isooctane started the product crystallization. The product can be recrystallized from ethyl acetate if needed. Chemical and optical purity of the final product was determined by the analytical methods described in the Analysis section.

Analysis

Optical densities were measured on a Shimadzu UV-160 spectrometer.

Quantification of (2,5)-hexanedione (**1**), 5-hydroxyhexane-2-one (**2**) and (2,5)-hexanediol (**3**) was carried out on an Agilent HP-5890A gas chromatograph with a Permabond Carbowax 20M column (50 m × 0.32 mm i.d., Macherey-

Nagel, Dueren, Germany), at 210 °C, with a flame ionization detector and helium as the carrier gas (1.5 bar). To minimize the injection error (1,5)-pentanediol (**4**) was used as an internal standard. Typical retention times were (**1**) 4.36 min, (**2**) 4.89 min, (**3**) 5.82 min, (**4**) 8.18 min.

To determine the enantio- and diastereoselectivity of all diastereomers the analytes had to be derivatized with trifluoroacetic acid anhydride prior to analysis. Each aqueous sample (300 μL) was extracted twice with 300 μL $CHCl_3$. Trifluoroacetic acid (100 μL) was added to the organic phase and incubated at 70 °C for 30 min. The sample was evaporated to dryness and resolved in 500 μL $CHCl_3$.

Determination of enantio- and diastereomeric excess was performed on a 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). Typical retention times were (*R*,*R*)-(**3**) 21.45 min, (*S*,*S*)-(**3**) 22.03 min, meso-(**3**) 25.05 min.

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