ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Characterization of a Whole-Cell Catalyst Co-Expressing Glycerol Dehydrogenase and Glucose Dehydrogenase and Its Application in the Synthesis of L-Glyceraldehyde

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ABSTRACT: A whole-cell catalyst using *Escherichia coli* BL21(DE3) as a host, co-expressing glycerol dehydrogenase (GlyDH) from *Gluconobacter oxydans* and glucose dehydrogenase (GDH) from *Bacillus subtilis* for cofactor regeneration, has been successfully constructed and used for the reduction of aliphatic aldehydes, such as hexanal or glyceraldehyde to the corresponding alcohols. This catalyst was characterized in terms of growth conditions, temperature and pH dependency, and regarding the influence of external cofactor and permeabilization. In the case of external cofactor addition we found a 4.6-fold increase in reaction rate caused by the addition of 1 mM $NADP^+$. Due to the fact that pH and temperature are also factors which may affect the reaction rate, their effect on the whole-cell catalyst was studied as well. Comparative studies between the whole-cell catalyst and the cell-free system were investigated. Furthermore, the successful application of the whole-cell catalyst in repetitive batch conversions could be demonstrated in the present study. Since the GlyDH was recently characterized and successfully applied in the kinetic resolution of racemic glyceraldehyde, we were now able to transfer and establish the process to a whole-cell system, which facilitated the access to L-glyceraldehyde in high enantioselectivity at 54% conversion. All in all, the whole-cell catalyst shows several advantages over the cell-free system like a higher thermal, a similar operational stability and the ability to recycle the catalyst without any loss-of-activity. The results obtained making the described whole-cell catalyst an improved catalyst for a more efficient production of enantiopure L-glyceraldehyde.

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Introduction

During the last decades much attention has been payed to the enzymatic production of chiral building blocks, like for example hydroxy esters, hydroxy acids, or chiral alcohols (Ghisalba et al., 2009). Oxidoreductases are high-potential biocatalysts because of their use in asymmetric oxidations as well as their ability to catalyze the stereo- and regioselective reduction of a variety of carbonyl compounds. Particularly, these enzymes have been used successfully in the production of chiral hydroxy acids (Vasic-Racki et al., 1989), amino acids (Bommarius et al., 1998; Galkin et al., 1997; Menzel et al., 2004), steroids (Crocq et al., 1997), and alcohols (Goldberg et al., 2007; Gröger et al., 2004; Liese et al., 1998; Pollard et al., 2006; Röthig et al., 1990; Schubert et al., 2001; Wills and Hannedouche, 2002). The produced chiral building blocks can act as key intermediates in the production of pharmaceuticals, fine chemicals, or natural products (Breuer et al., 2004; Patel, 2006; Pesti and DiCosimo, 2003; Rouhi, 2004).

Enantiopure glyceraldehyde is an example for a small chiral building block of great interest because of the wide applicability of its structural motif. Recently, it has been successfully used in the synthesis of, for example, enantiopure 4-(dihydroxyalkyl)-β-lactams (antibiotic) (Areces et al., 2007) or 8a-*epi*-swainsoine (anticancer drug; Bi and Aggarwal, 2008). Both glyceraldehyde enantiomers have been synthesized chemically by different approaches starting from chiral pool compounds (Andrews et al., 1981; Baer and Fischer, 1939; Hubschwerlen, 1986; Hubschwerlen et al., 1995; Jurczak et al., 1986; Schmid and Bryant, 1995). The step economy and accumulated waste streams of the existing procedures are however not very favourable for industrial applications despite the achieved yields of the individual steps. This is due to the synthesis design using protecting groups which generate stoichiometric waste, toxic reagents, many synthetic steps, and organic solvents. With regard to environmental improvements and cost reductions, new direct enzymatic routes for the preparation of enantiopure glyceraldehyde are attractive. In a previous paper, we could successfully report such an enzymatic route (Richter et al., 2009) by applying the glycerol dehydrogenase (GlyDH) from *Gluconobacter oxydans* in the kinetic resolution of racemic glyceraldehyde, producing enantiopure L-glyceraldehyde on preparative scale.

However, in terms of efficiency, the need for isolated enzymes and as well the requirement for addition of expensive cofactor $NADP^+$ is disadvantageous. Therefore, a regeneration of the reduced cofactor is necessary by either a second substrate or a second enzyme together with a second substrate (Chenault and Whitesides, 1987; Geueke et al., 2003; Hummel, 1999; Weckbecker et al., 2010). To overcome the problem of cofactor regeneration and to improve and simplify the process, a simultaneous expression of two or more enzymes in a single cell is a promising approach, which has been shown by numerous literature known processes using whole-cell catalyst (Goldberg et al., 2007; Gröger et al., 2004, 2006a,b; Kataoka et al., 1999; Kosjek et al., 2008; Patel et al., 2004; Schroer et al., 2007; Uzura et al., 2009; Weckbecker and Hummel, 2004).

Different enzymes have already been applied in the regeneration of either the reduced or the oxidized cofactor in cell-free or whole-cell systems (Ernst et al., 2005; Hummel, 1999; Kosjek et al., 2008; Moore et al., 2007; Weckbecker and Hummel, 2004). For the regeneration of the oxidized cofactor we used glucose dehydrogenase (GDH) from *Bacillus subtilis*, an enzyme which can regenerate both NADH and NADPH (Fujita et al., 1977; Hilt et al., 1991; Weckbecker and Hummel, 2004, 2005). So far, it has been successfully applied in NADH-dependent biotransformations (Hanson et al., 1999) as well as in a number of NADPH-dependent processes. Examples are given by coupling with a (*R*)-specific alcohol dehydrogenase in the production of different chiral alcohols (Gröger et al., 2006a; Weckbecker and Hummel, 2005), or the production of 4-chloro-3-oxobutanoate where it was used in combination with an aldehyde dehydrogenase (Kataoka et al., 1999). These examples display the broad applicability of the GDH in combination with various oxidoreductases.

Based on the process established for the cell-free biotransformation using GlyDH and GDH in the production of L-glyceraldehyde, we constructed a whole-cell catalyst expressing both enzymes in parallel. Furthermore, we characterized the catalyst in terms of pH, temperature, storage and operational stability, and other parameters, and compared the whole-cell catalyst to the cell-free system. We were able to demonstrate that like the cell-free system, the whole-cell catalyst can also be applied successfully in the kinetic resolution of racemic glyceraldehyde producing enantiopure L-glyceraldehyde.

Materials and Methods

General

If not stated otherwise all chemicals were purchased from Sigma–Aldrich, Buchs, Switzerland. Centrifugations were carried out using the centrifuges RC5BPlus (Sorvall, Waltham, USA), Mikro22, and Rotina 35 R (Hettich, Tuttlingen, Germany). Restriction enzymes were purchased from Fermentas, St. Leon-Rot, Germany. The studied GlyDH and GDH are commercially available at evocatal GmbH (evo-1.1.190 and evo-1.1.060).

Construction of the Whole-Cell Catalyst

For cloning of the *glydh* gene, the plasmid pNR-1 was used as template for PCR introducing the restriction sites *Nde*I and *Kpn*I (up: 5′-GGAATTCCATATGGCATCCGACACCATCCGC-3′ down: 5′-GGGGTACCTCAGTCCCGTGCCGGGGG-3′). The amplification was performed using standard PCR techniques, the created *Nde*I–*Kpn*I fragment was subcloned into the vector pGDH1. This vector is based on the commercially available pETDuet-1. The *gdh* gene was amplified using genomic DNA from *B. subtilis* as template and primers with recognition sites for the restriction enzymes *Eco*RI and *Not*I (up: 5′-CCGGAATTCATGTATCCGGATTTAAAAGGAAAAGTCG-3′, down: 5′-ATAGTTTAGCGGCCGCTTAACCGCGGCCTGCCTG-3′). The resulting construct was named pNR-2 (Fig. 1).

Cultivation of the Whole-Cell Catalyst in Shaking Flasks

Escherichia coli BL21(DE3) cells carrying the recombinant plasmid (pNR-2) were cultivated overnight at 37°C in 5 mL LB medium (Bertani, 1951), containing 100 μg/mL ampicillin. These cultures were used to inoculate different amounts of LB medium, TB medium (24 g/L yeast extract, 12 g/L casein hydrolysate, 5 g/L glycerol in 100 mM KP_i buffer pH 7), autoinduction (AI) medium (TB medium, 2 g/L lactose, 0.5 g/L glucose), and M9 (4 g/L glucose, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 70 g/L $Na_2HPO_4 \cdot$

$2H_2O$, 30 g/L KH_2PO_4, 5 g/L NaCl, 10 g/L NH_4Cl in distilled water) containing 100 μg/mL ampicillin for expression in shaking flasks at a final concentration of 0.05 optical density at 600 nm (OD_{600}). The cultures were grown at 37°C. When the OD_{600} reached a value between 0.5 and 0.7 the production of the recombinant GlyDH and GDH were induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.3 mM. The cultures were shaken for 4 or 20 h at 25, 30, or 37°C, and harvested by centrifugation. For the detailed characterization of the whole-cell catalyst cells cultivated in LB medium at 25°C were used.

High-Density Fermentation (HDF)

Fermentation of *E. coli* BL21(DE3) cells carrying the recombinant plasmid (pNR-2) was performed in a 40 l bioreactor (Infors AG, Einsbach, Germany) in a 15 L scale.

The components of the batch medium are as follows: 0.2 g/L NH_4Cl, 2 g/L $(NH_4)_2SO_4$, 13 g/L KH_2PO_4, 10 g/L K_2HPO_4, 6 g/L $NaH_2PO_4{\cdot}H_2O$, 3 g/L yeast extract, 2 g/L glucose, 1 g/L $MgSO_4{\cdot}7H_2O$, 0.25% vitamin solution (0.1 g/L riboflavin, 10 g/L thiamine, 0.5 g/L nicotinic acid, 0.5 g/L pyridoxine, 0.5 g/L Ca-phanthotenate, 0.001 g/L biotin, 0.002 g/L folic acid, 0.01 g/L cyanocabalamine), 0.16% micronutrient solution (10 g/L $CaCl_2{\cdot}2H_2O$, 0.5 g/L $ZnSO_4{\cdot}7H_2O$, 0.25 g/L $CuCl_2{\cdot}2H_2O$, 2.5 g/L $MnSO_4{\cdot}H_2O$, 1.75 g/L $CoCl_2{\cdot}6H_2O$, 0.125 g/L H_3BO_3, 2.5 g/L $AlCl_3{\cdot}6H_2O$, 0.5 g/L $Na_2MoO_4{\cdot}2H_2O$, 10 g/L $FeSO_4{\cdot}7H_2O$), 0.1 g/L thiamine in distilled water. Additionally, a fed medium was used which consists of the same components as the batch medium but differs in the following concentrations: 18 g/L yeast extract, 600 g/L glucose, 10 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L thiamine.

After sterilization of the reactor including the batch medium the HDF was started by an inoculation of 1% (v/v) with pre-culture grown overnight at 37°C. For optimal growth, the cells were cultivated under glucose limiting conditions. The addition of fed medium was done according to an optimized protocol for *E. coli* cells at 30°C. H_3PO_4 was used to keep the pH at a constant level (pH 7.0), the oxygen feed was controlled by increasing agitation speed and airflow to keep it over 30%. Then, the protein expression was induced after 27 h by an IPTG concentration of 2 mM. After induction, the temperature was shifted from 30 to 25°C. Temperature, pH, and oxygen feed were controlled automatically by computer. After an incubation time of 41 h, the cells were harvested by centrifugation.

Preparation of Cell-Free Extracts

The bacterial culture was harvested by centrifugation at 17,000*g* for 20 min at 4°C. A cell suspension (20%) was prepared in 100 mM triethanolamine buffer (TEA) pH 7.0. Cells were disrupted by two sonification cycles of 2 min (40% power output) with cooling periods in-between. The lysed cells were centrifuged at 17,000*g* for 30 min at 4°C, and the supernatant was used for determination of GlyDH and GDH activity. Protein concentrations were determined according to Bradford using BSA as a standard (Bradford, 1976).

Real-Time PCR

For RT-PCR analysis cells were cultivated as described; only the point of induction was shifted to an OD_{600} of 0.3 and cells were incubated at 25°C until a final OD_{600} of 0.9 was reached. Total RNA was isolated using the RNeasy Mini Kit including the optional DNA digestion step (Qiagen, Hilden, Germany). An additional DNA digestion was performed with the RQ1 RNase-free DNase Set (Promega, Madison, USA). RNA concentrations were determined using an Eppendorf BioPhotometer equipped with a Tray Cell 105.810 UVS (Hellma, Jena, Germany). A 150 ng of total RNA in a reaction volume of 20 μL were used for reverse transcription and subsequent RT-PCR using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). The temperature program was adjusted according to manufacturers' instructions (annealing temperature 58°C, 30 cycles) in a Mastercycler epgradient S with realplex^4 module (Eppendorf). For amplification, the following primers were used: *glydh* (up: 5′-CCTGCTG ACAGGCAAGATGAAC-3′, down: 5′-GAGGTACTTCTCGAAATTCGGCTTC-3′), *gdh* (up: 5′-GCTGACCCTAAACAGAAAGCTGATG-3′, down: 5′-GAATAACGTGATGCCTGTGACGTAG-3′) and *bla* as internal standard (up: 5′-TAACTACGATACGGGAGGG-CTTACC-3′, down: 5′-GGA TAAAGTTGCAGGACCA-CTTCTG-3′). The given data are results of at least three measurements.

Spectrophotometrical Assays for the Determination of the GDH and GlyDH Activity

Continuous assays using UV absorbance at 340 nm were employed to monitor the NADPH concentration during reduction or oxidation catalyzed by GlyDH or GDH. One unit of activity was defined as the amount of enzyme which catalyzes the oxidation of 1 μmol NADPH per minute (GlyDH) or reduction of 1 μmol $NADP^+$ (GDH) under standard conditions (30°C, pH 7). For the determination of GlyDH activity, racemic glyceraldehyde was used as standard substrate. The assay mixture contains 970 μL substrate solution (10 mM substrate in 100 mM TEA buffer pH 7), 20 μL NADPH (12.5 mM) in distilled water, and 10 μL enzyme solution. For the determination of GDH activity D-glucose was used as substrate, standard conditions were 30°C and pH 7.0. The assay mixture contains 970 μL substrate solution (100 mM substrate in 100 mM TEA buffer pH 7), 20 μL $NADP^+$ (100 mM), and 10 μL enzyme solution. Reactions were started by addition of the enzyme solution and the amount of NADPH was measured over 1 min.

Determination of Initial Rates of the Whole-Cell Catalyst

For the use in whole-cell biotransformations 5 mg cells (wet weight) were suspended per mL buffer (100 mM TEA, pH 7). A total of 500 μL/mL of the cell solution and 500 μL/mL of substrate solution (20-40 mM substrate, 200 mM D-glucose in 100 mM TEA, pH 7.0) were mixed. Hexanal (40 mM) was used as standard substrate for determination of initial rates. Biotransformations were carried out at 30°C and 800 rpm. Samples of 100 μL were taken periodically and diluted with 100 μL of internal standard (2.3 mM octanol) and 800 μL ethyl acetate. After mixing and centrifugation, the organic phases were analyzed for the production of hexanol using the gas chromatograph (GC-17A; Shimadzu, Duisburg, Germany) equipped with the column CP-Chirasil-DEX (Varian, 25 m × 0.25 mm ID). The temperature program included: 60°C for 2 min, 5°C/min to 105°C, and 10°C/min to 130°C. To examine alterations in initial rates, different parameters such as pH, temperature, cofactor concentration, or permeability of the cells were varied and the initial rates were determined using standard conditions. The activity of the whole-cell catalyst was determined by measuring product formation at different times followed by calculation of the initial product formation rate per gram wet cells. The activity of whole-cell catalyst is expressed as U which means μmol product formed per minute and the cell-specific activity is expressed as U/g cell wet weight.

Recycling of the Whole-Cell Catalyst

For recycling studies of the whole-cell catalyst transformations with freshly harvested cells were performed. Cells (60 mg wet weight) per mL were suspended in substrate solution (20 mM hexanal, 200 mM D-glucose in 100 mM TEA buffer pH 7). Conversions were carried out at 30°C and 800 rpm. After 0, 15, 30, and 45 min samples were taken and analyzed by GC as described. After 45 min conversion, the cells were separated by centrifugation and the supernatant was discarded. The cells were suspended in fresh substrate solution and the conversion over 45 min was determined again.

Whole-Cell Biotransformation

For the kinetic resolution of glyceraldehyde, the reaction mixture contained racemic glyceraldehyde (4 g, 0.18 M), glucose (5.28 g, 0.12 M), $NADP^+$ (0.1 g, 0.5 mM) in 250 mL of TEA buffer (100 mM, pH 7) and the whole-cell catalyst (2 g wet weight). Samples of 50 μL were taken periodically and diluted either with trifluoroacetic acid (950 μL) for HPLC analysis to determine the conversion or with ethyl acetate (75 μL) for GC analysis to determine the enantiomeric excess (EE). GC samples were derivatized for 30 min with dioxane (75 μL), acetic anhydride (50 μL), and pyridine (5 μL). Concentrations of glyceraldehyde and glycerol were determined by using an Agilent 1100 HPLC equipped with a CS organic acid resin column (300 mm × 8 mm) with 5 mM trifluoroacetic acid (0.7 mL/min) at room temperature. The enantiomeric ratio for glyceraldehyde was determined by using a HP 6890 GC with a Varian CP-Chirasil-Dex CB column (25 m × 0.32 mm). The temperature was kept constant at 108°C over a period of 20 min.

Results

Construction of Recombinant *E. coli* Cells Co-Expressing GlyDH and GDH

For co-expression of the *glydh* and *gdh* genes in *E. coli* BL21(DE3), the plasmid pNR-2 has been constructed (Fig. 1). The different specific activities of the pure investigated enzymes (GlyDH 39 U/mg and GDH 295 U/mg; Richter et al., 2009; Weckbecker and Hummel, 2005) led to the chosen arrangement of the genes in the commercial vector pETDuet-1. Based on the fact that no transcription terminator is located between the two MCS, *gdh* was cloned into MCS-1 and *glydh* into the MCS-2.

As next step the efficient co-expression of the whole-cell catalyser cultivated in LB medium at 25°C (standard conditions) was investigated in detail using both RT-PCR and spectrophotometric activity assays. Since it was the aim to regulate the activity of the GlyDH and GDH by controlling the transcripts of the corresponding genes, transcript levels of the *glydh* and *gdh* gene were analyzed by RT-PCR. The studies revealed that the amount of *glydh* gene transcript is 10-fold higher compared to the *gdh* gene. Due

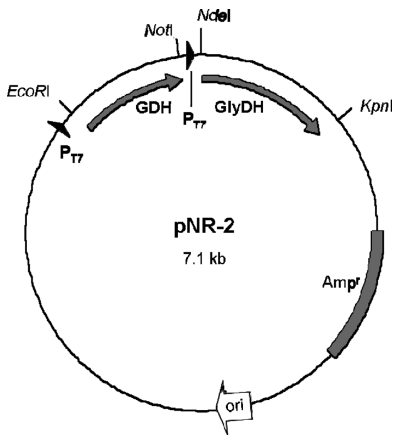


Figure 1. Structure of the plasmid pNR-2 derived from pETDuet-1 containing the genes for GlyDH and GDH. Additionally the plasmid contains an amp^r and two T7 promoters.

to the 7.6-fold higher specific activity of the GDH on transcript level the difference could be adjusted. Therefore, further experiments concerning the enzyme activity were performed additionally.

The functional expression of both enzymes was determined by measuring the activities in cell-free extracts. The GlyDH activity was found to be 22.3 U/mg of crude protein which was 3.1-fold higher than the activity determined for the GDH (7.18 U/mg of crude protein). Accordingly, the results of transcription levels could be confirmed by the analysis of the expression level, demonstrating that a co-expression with activities in a similar range could be successfully performed expressing pNR-2 in *E. coli* BL21(DE3). In the recent study, the constructed whole-cell catalyst has been characterized regarding different parameters.

Biotransformation of Hexanal

In order to study the influence of different factors on the activity of the whole-cell catalyst the reduction of hexanal to hexanol has been selected as standard reaction (Fig. 2). This reaction was selected as model due to a better applicability and a more reliable GC analysis for hexanal and hexanol.

Initial rates have been determined measuring the production of hexanol over 30 min at 30°C without cofactor addition. Figure 3 illustrates a typical time course for the determination of the initial rate under standard conditions. The slope has been used for the calculation of the activity of the whole-cell catalyst (U/g). The control (empty vector) showed no activity at all, whereas the whole-cell catalyst on the contrary showed an activity of 27.2 ± 7.3 U/g (Fig. 3).

For a better comparison of the whole-cell and the cell-free systems, initial rates for both systems were determined using the same amount cell-free extract obtained by disrupting the whole-cell catalyst by sonification. The activity of the cell-free system was 1.5-fold higher than using the whole-cell system, indicating a better access for substrate and cofactor to the enzymes involved in the reaction (data not shown).

Using these standard conditions different parameters and their influence on the whole-cell catalyst have been investigated.

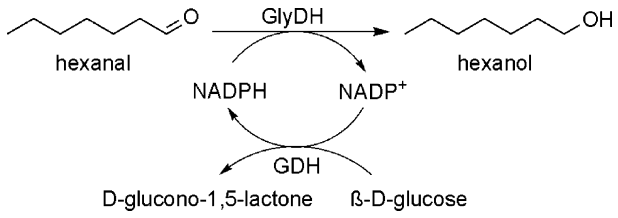


Figure 2. Scheme of the reaction catalyzed by the whole-cell catalyst. Hexanal is reduced by the GlyDH while the GDH simultaneously regenerates the cofactor catalyzing the oxidation of β-D-glucose.

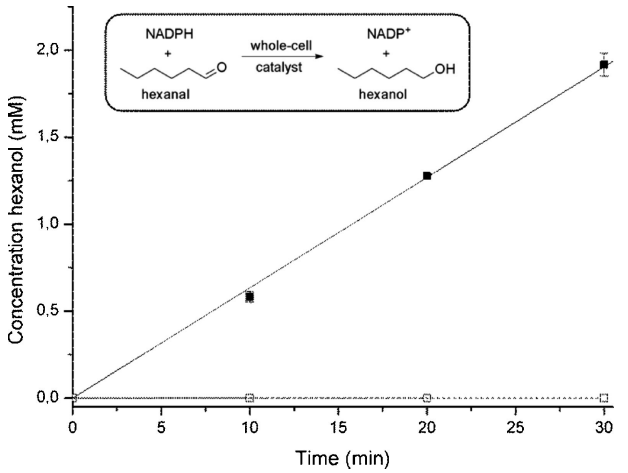


Figure 3. Determination of the initial rates in the conversion of 20 mM hexanal. The figure shows a typical time curve for the production of hexanol over a period of 30 min using per mL either 2.5 mg whole-cell catalyst (■) or 2.5 mg of cells expressing the empty vector ("□"). Standard deviations are given in the diagram, hexanol concentrations were measured by means of gas chromatography.

Effect of Growth Conditions and Media on the Expression Level of the GlyDH and GDH

Based on economical reasons, an optimal expression and especially an ideal ratio of the two co-expressed genes is of major importance. In addition to molecular methods, variation of growth conditions and media is a way to influence the expression levels of the corresponding enzymes. Therefore, different growth conditions and a variety of media have been tested to optimize the expression levels of the two co-expressed enzymes.

The corresponding results are summarized in Table I, showing that best GlyDH activities were observed expressing the whole-cell catalyst at 25°C in LB, AI, and M9 medium. In contrast to the GlyDH, the GDH activity increased with higher incubation temperatures. In general, a retarded expression caused by suboptimal growth conditions such as reduced temperature and minimal medium (M9) led to an increased GlyDH activity, whereas better GDH activities were reached at optimal growth conditions like 37°C. For instance the best GlyDH activity was reached with cells cultivated at 25°C in minimal medium (M9). This crude extract showed a 1.5-fold higher activity in the reduction of *rac*-glyceraldehyde compared to LB medium. For the GDH, the best activity was reached at an expression at 37°C in LB medium for 4 h, where a 1.5-fold enhancement of activity, compared to 25°C and 20 h, could be achieved.

Regarding the activity and ratio of both enzymes involved, LB medium at different temperatures and the HDF showed best results. Based on the observed spectrophotometrical results, the initial rates of the whole-cell catalyst cultivated under these four different conditions in the reduction of hexanal have been comparatively studied.

Table I. Effect of growth conditions and media on the expression levels of GlyDH and GDH.

Medium	Temperature/incubation time	Relative GlyDH activity (%)	Relative GDH activity (%)	Ratio (GlyDH/GDH)
LB medium	25°C/20 h	100	100	3.1*
LB medium	30°C/4 h	46.2	91.1	1.6*
	30°C/20 h	80.7	105.8	2.4
LB medium	37°C/4 h	35.0	150.4	0.7*
	37°C/20 h	30.9	62.7	1.5
TB medium	25°C/20 h	14.8	2.8	18.1
Autoinduction medium	25°C/20 h	143.9	66.9	6.7
M9 medium	25°C/20 h	147.1	114.2	4.0
High-density fermentation	25°C/41 h	43.5	105.8	1.3*

Activities are given in relation to LB medium at 25°C (100%), 100% equates to 22.3 U/mg crude protein for the GlyDH and 7.18 U/mg crude protein for the GDH. All activities have been measured with *rac*-glyceraldehyde (GlyDH) and D-glucose (GDH) as substrates using the described spectrophotometrical assays with cell-free extract of the whole-cell catalyst

*Cells cultivated under these conditions were used for further investigations regarding initial rates in the reduction of hexanal to hexanol (Fig. 4).

The results are shown in Figure 4 (diamond). Best conversions were reached with the cells cultivated at 25°C in LB medium (standard conditions, black) where the activity ratio of GlyDH and GDH was 3.1. A cultivation at 30°C for 4 h lead to an activity ration of 1.6 and an initial rate of 58.0% in the reduction of hexanal compared to standard conditions. The GDH activity of the whole-cell catalyst cultivated at 37°C is higher than the GlyDH activity under same condition resulting in a ratio of 0.7. Cells cultivated under these conditions reached an initial rate of 96.5% compared to the conversion velocity under standard conditions. A cultivation of the whole-cell catalyst under high-density conditions led to a ratio of 1.3 in activity levels of GlyDH and GDH. These cells showed an initial rate of 84.1% of the rate determined after an expression at 25°C.

Thus, by the use of different growth conditions and media, the ratio of the two co-expressed enzymes could be varied from 0.7 (LB medium, 37°C, 4 h) to 18.1 (TB medium, 25°C, 20 h). However, the total activity of the involved enzymes is also a crucial parameter. The observed results regarding total performance of the catalyst in the reduction of hexanal indicates that a good ratio combined with high activities of GlyDH and GDH is necessary for the construction of an efficient catalyst. This point is

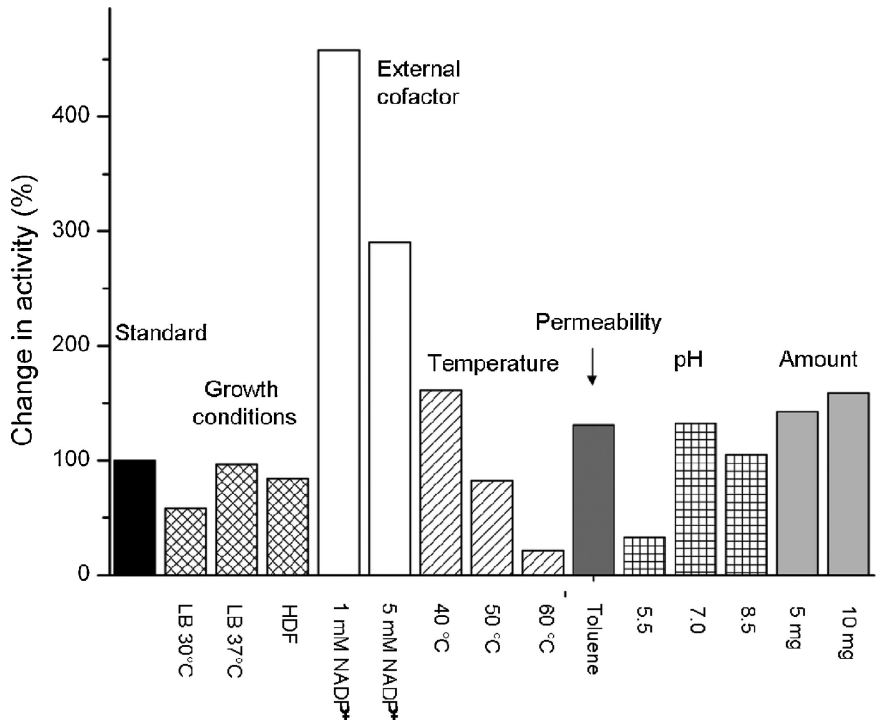


Figure 4. Influence of different parameters on the initial rate in the conversion of hexanal by the whole-cell catalyst. The initial rate under standard condition has been set to 100% (black). Alterations in initial rates by growth conditions (diamond), addition of external cofactor (white), variation of temperature (stripped), permeability (gray), pH (plaid), and amount of whole-cell catalyst (light gray) are displayed. All the data shown are the results of at least three independent measurements with standard deviations (>10%).

emphasized by the fact that a good ratio of 1.3 and a medium GlyDH activity (HDF) led to a comparable performance of the whole-cell catalyst that a medium ratio 3.1 and a high-GlyDH activity (LB medium, 25°C).

Influence of External Cofactor on Whole-Cell Biotransformation

The cofactor concentration has a major impact on enzyme activity. By using whole-cell systems, the intracellular cofactor level can be a limiting factor. Under normal growth conditions, the $NADP^+$ and NADPH level of *E. coli* is in the range of 3.62 and 2.42 nmol/mg dry weight (Lilius et al., 1979), meaning that the sum of the intracellular concentration of $NADP^+$ and NADPH is approximately 0.4 mM (Walton and Stewart, 2004). In consideration of the fact that the investigated enzymes show K_m values for NADPH of 0.04 mM (GlyDH, determined with *rac*-glyceraldehyde as substrate; Richter et al., 2009) and 0.025 mM for $NADP^+$ (GDH, determined with D-glucose as substrate; Weckbecker, 2005) optimal activities should be obtainable. However, due to differences in growth conditions and an often noticeable loss of internal cofactor by permeabilization effects, optimal activities are difficult to achieve. Therefore, experiments concerning the influence of the addition of different amounts of $NADP^+$ (1 and 5 mM) on the initial rates were studied. The results indicate that the addition of external cofactor led to a considerable increase in enzyme activity resulting in a faster conversion of hexanal in comparison to the experiment under standard conditions without cofactor addition (Fig. 4). Surprisingly, the addition of 1 mM $NADP^+$ elevated the initial rate by factor 4.6 whereas the addition of 5 mM $NADP^+$ led to a lower improvement (2.9-fold) under standard conditions. This inactivation in the presence of higher cofactor concentrations can be explained by a substrate-excess inhibition of the GlyDH.

Effect of Toluene Treatment on Whole-Cell Biotransformation

As a second parameter the permeabilization of the cells by toluene has been studied. Therefore, the cells were incubated with 1% (v/v) toluene for 1 h at 30°C under shaking at 800 rpm. The treatment was followed by the determination of initial rates. Figure 4 (gray) illustrates that a treatment with toluene increases the initial rate by factor 1.3. This demonstrates that a permeabilization of the cells results in an improved access of the substrate to the enzymes leading to an enhanced initial rate in the biotransformation of hexanal.

Dependence of Temperature and pH on the Activity and Stability of the Whole-Cell Catalyst

Since the influence of temperature and pH on enzyme activity and stability is often a limiting parameter in technical applications, studies concerning the dependence of temperature and pH on the whole-cell catalyst were performed. Furthermore, the temperature stability of the whole-cell catalyst and the cell-free system were examined comparatively.

Regarding temperature dependence, the whole-cell catalyst showed optimal activity at 40°C with a 1.6-fold higher initial rate compared to standard conditions (Fig. 4). Elevated temperatures decreased the activity of the whole-cell catalyst to approximately 80% residual activity at 50°C or 20% residual activity at 60°C, respectively. In addition, the stability under these elevated temperatures was reduced as well which can be seen as a result of the measurable inactivation of the catalyst after 10 min.

The pH dependence was investigated at three different pH values (pH 5.5, 7.0, and 8.5) and compared to the standard conditions at pH 6.8 (Fig. 4). At pH 7.0 the catalyst showed optimal activity (132.1%), whereas at pH 8.5 no improvement could be detected at all. Under slightly acidic conditions (pH 5.5), the whole-cell catalyst lost approximately 70% of its activity. Because of the importance of catalyst stability in technical applications, temperature-dependent inactivation over time of the whole-cell catalyst and the cell-free system were investigated. Studies were performed incubating both preparations at 30°C and under shaking (800 rpm) in buffer (100 mM TEA, pH 7) and under conditions where *rac*-glyceraldehyde was converted over 48 h. For studies regarding the cell-free system the same amount cell-free extract of the whole-cell catalyst was used. Residual activities after 4, 24, and 48 h were determined using the standard assay for initial rates.

As shown in Figure 5, after incubation in buffer the whole-cell catalyst is completely stable over the investigated period of time, whereas the cell-free system showed a significant inactivation, revealed by a residual activity of only 18.9% after 48 h. In contrast to the observed stabilities in buffer, both preparations show a significant decrease in activity when incubated under process conditions. After an incubation of 4 h residual activities of 32.4% using the cell-free system and 40.9% with the whole-cell catalyst were determined. The residual activities further diminish over the 48-h incubation period. At that time, the cell-free system shows a loss of 73.2% of its activity, while the whole-cell catalyst loses 83.3% of its activity, indicating that over time an inactivation in both enzyme preparations is visible while simultaneously converting *rac*-glyceraldehyde.

Comparing the observed results under process and storage conditions, both systems show a more vigorous decrease in residual activity under process conditions. Concerning the cell-free system, similar residual activities were observed under both investigated conditions, while in contrast the activity of the whole-cell catalyst is drastically decreased under conditions where *rac*-glyceraldehyde is converted.

Nevertheless, after 48 h both systems still show activity, and due to the fact that enzyme stability is a very crucial parameter for technical applications and processes, any

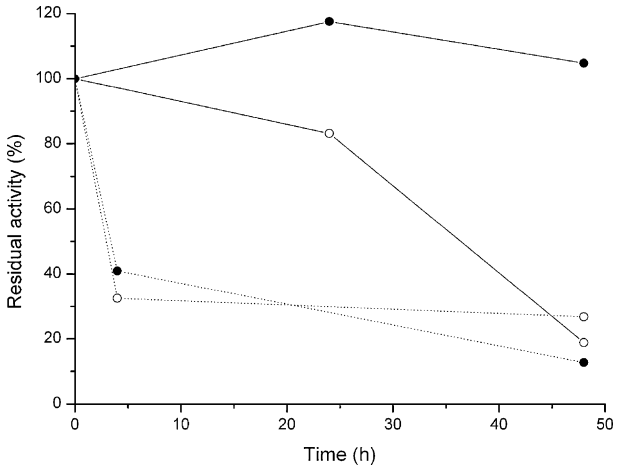


Figure 5. Thermal and operational stability of the whole-cell catalyst (●) in comparison with the cell-free system (○). The effect of temperature on both preparations stored in buffer (solid line) and under operational conditions (dotted line) over a period of 48 h at 30°C was investigated. Operational stability was measured under conditions where substrate (*rac*-glyceraldehyde) was converted by the catalyst over the investigated period. For the determination of the residual activity the initial rates were measured under standard conditions. All the data shown are the results of at least three independent measurements with standard deviations (<10%).

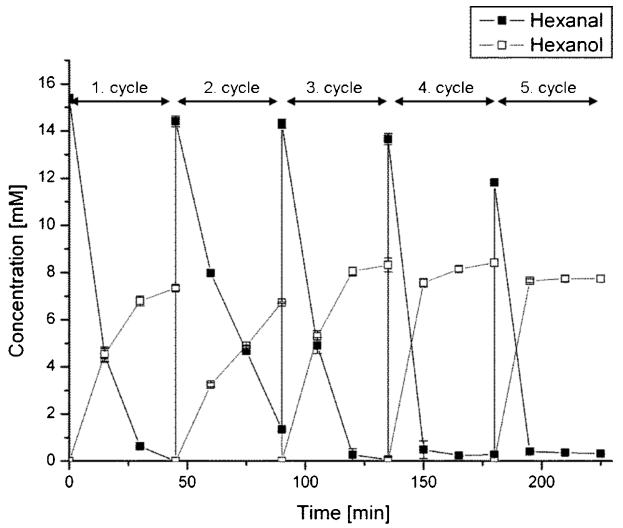


Figure 6. Result of a repetitive conversion of hexanal (15 mM) by the whole-cell catalyst. The figure shows the development of hexanal and hexanol concentrations over 225 min where 5 cycles were performed. After each conversion the samples were withdrawn, the supernatant was discarded and the whole-cell catalyst was suspended in fresh substrate solution. Standard deviations are indicated in the diagram.

improvement in stability is important. Herein, we could show that the whole-cell catalyst is significantly more stable for long-term applications than the cell-free system incubated in buffer. But regarding the stability while *rac*-glyceraldehyde is converted, both systems show residual activities of 26.8% for the cell-free system and 16.7% for the whole-cell catalyst operational stabilities in the same dimension.

Recycling of the Whole-Cell Catalyst

Recycling of the whole-cell catalyst would generously facilitate technical applications in terms of efficiency and cost factors. For this reason, we investigated the ability of using the whole-cell catalyst in repetitive batch conversions. Studies were performed by measuring the reduction of hexanal to hexanol as a model system over a period of 45 min. After this incubation time, samples were withdrawn and the supernatant was discarded. By suspending the cells in fresh substrate solution the next cycle was started. In the reduction of hexanal the whole-cell catalyst showed 100% conversion of hexanal, while simultaneously only 50% of product hexanol was formed. But due to the fact, that this reaction was chosen as model reaction the suboptimal product formation of the reaction was not further investigated.

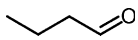
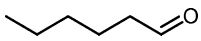
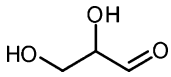
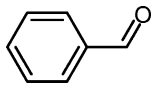
First experiments showed that cells which were exposed to several freezing and unfreezing cycles were not recyclable. Indicating that this treatment led to a high level of permeabilization, this thesis was proven by studies where stored cells were used for recycling experiments. The results showed that the normal conversion in the first cycle was followed by a massive loss-of-activity in the second cycle and no detectable activity in the third (data not shown). According to this observation, freshly harvested and washed cells have been used for the experimental setup to investigate the ability to recycle the whole-cell catalyst.

Figure 6 illustrates that under these conditions a recycling of the whole-cell is possible for at least five times without any loss in activity. In the third cycle, the reaction rate even increased and the maximal conversion was reached after a reaction time of approximately 30 min compared to the first cycle where a product formation of 50% was reached after 45 min. Further increase of activity was observed in cycles 4 and 5 where the reaction rate increases even more and the conversion was completed after only 15 min. The observed results lead to the suggestion that the increase in reaction rate is a result of the ongoing permeabilization of the catalyst caused by compounds in the reaction mixture. This thesis is emphasized by the observation that cells which are partially permeabilized by a toluene treatment showed a faster conversion than non-permeabilized ones. Along with the results, regarding the use of frozen cells, it becomes apparent that permeabilization leads to an increased reaction rate, but at a certain degree of permeabilization, a recycling of the whole-cell catalyst is not feasible anymore.

Substrate Spectrum

For the purified GlyDH the activity towards a variety of different substrates was determined (Richter et al., 2009). From this substrate spectrum, two aliphatic, one branched-

Table II. Comparison of the substrate specificity of the whole-cell catalyst and the purified enzyme.

Substrate	Structure	Relative activity whole-cell catalyst (%)	Relative activity purified enzyme (%)
Butanal		17.9	73.0
Hexanal		100	100
rac-Glyceraldehyde		78.8	122.6
Benzaldehyde		72.2	43.3

Activities are given in relation to hexanal (100%) (100% equates to 31.9 U/mg for the purified enzyme and 27.2 U/g). For the whole-cell catalyst initial rates with 20 mM substrate at 30°C were determined by means of gas chromatography.

chained, and one aromatic substrate were chosen for the comparison of the substrate specificity in the reduction of purified enzyme and whole-cell catalyst (Table II).

For both preparations, activities were compared to the standard substrate hexanal (specific activity for the purified enzyme 31.9 U/mg). All selected substrates were accepted by the whole-cell catalyst and the purified enzyme, however, revealing different relative activities towards the different substrates. For example, butanal was converted at a significantly lower activity by the whole-cell catalyst (17.9% related to hexanal) than using purified enzymes which showed an activity of 73.0% compared to hexanal. The reductions of benzaldehyde and glyceraldehyde were catalyzed with better activities using the whole-cell catalyst. These differences can be caused either by a different access to the enzyme by the diverse substrates due to the variable catalyst preparations or due to the existence of interfering enzymes in the crude extract catalyzing unspecific reactions. In general, there are some differences detectable regarding the activities towards the different substrates, but all in all the same substrates are accepted by the two different catalyst preparations (Table II).

Application of the Whole-Cell Catalyst in the Kinetic Resolution of Glyceraldehyde

Enantiopure glyceraldehyde is an interesting chiral building block which can be used as starting material for the synthesis of a variety of different fine chemicals, pharmaceuticals, and natural products (Areces et al., 2007; Bi and Aggarwal, 2008; Bull et al., 2007; Kumar et al., 2007). Therefore, we tried to transfer results regarding the cell-free system (Richter et al., 2009) to a whole-cell system (Fig. 7). Thus, preparative scale kinetic resolution was performed using the whole-cell catalyst. Due to the obtained results regarding the externally added cofactor we performed the kinetic resolution of racemic glyceraldehyde in the presence of 0.5 mM $NADP^+$. This concentration was chosen as a result of comparative conversions with 0.1, 0.5, and 1 mM cofactor (data not shown). The results revealed that with an addition of 0.5 mM the conversion proceeds in reasonable time.

Figure 8 illustrates a typical time curve for the kinetic resolution of racemic glyceraldehyde using the whole-cell catalyst. The developments of the glyceraldehyde and glycerol concentrations are displayed in the diagram. Additionally, the EE value of L-glyceraldehyde was measured over the investigated period of 60 min. A conversion of 54% and EE of 98% were achieved by using the whole-cell catalyst in the kinetic resolution of racemic glyceraldehyde. The reaction continues after a conversion of 50%. Therefore, the reaction has to be stopped at a conversion of approximately 54%. Moreover, a conversion without cofactor addition was possible, resulting in comparable selectivity. Drawbacks of the cofactor-free application are the requirement of a higher amount of catalyst and longer incubation times (data not shown). Nevertheless, we succeeded in transferring the kinetic resolution of racemic glyceraldehyde to a whole-cell system.

Discussion

In the present study, the successful construction and characterization of a whole-cell catalyst co-expressing GlyDH from *G. oxydans* and GDH from *B. subtilis* is presented. Moreover, the successful application of the whole-cell catalyst in the kinetic resolution of *rac*-

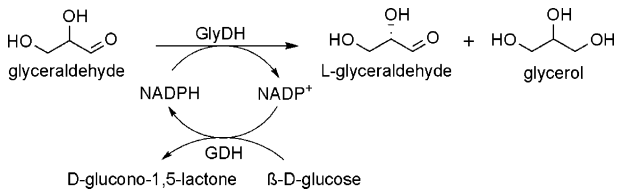


Figure 7. Scheme of the kinetic resolution of glyceraldehyde by the whole-cell catalyst combining GlyDH and GDH.

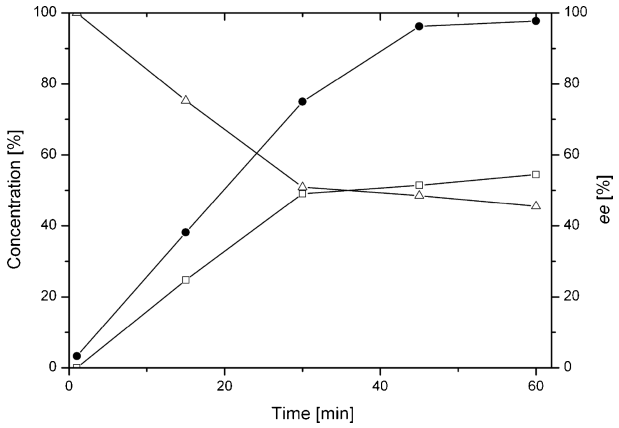


Figure 8. Kinetic resolution of racemic glyceraldehyde by the whole-cell catalyst. The development of glycerol (□) and glyceraldehyde (△) concentrations are measured over a period of 60 min; concentrations were determined by HPLC. The EE values were determined by means of chiral GC (●). The kinetic resolution was done using 2 g catalyst, 0.18 M *rac*-glyceraldehyde, 0.12 M glucose, and 0.5 mM $NADP^+$ in 250 mL 100 mM TEA pH 7.0.

glyceraldehyde producing enantiopure L-glyceraldehyde is reported in this paper.

An one plasmid strategy was applied cloning both genes into one plasmid for simultaneous expression of both proteins. We succeeded in generating a catalyst which showed activities in a similar range fulfilling an important issue regarding an efficient application of the catalyst (Gröger et al., 2006b; Menzel et al., 2004). Apart from the successful expression of both genes other parameters play an important role for the applicability of the catalyst. In terms of efficiency, the need of external cofactor and the ability of catalyst recycling are of primary focus.

We could show that the presented catalyst stored in buffer is more stable than the cell-free system. Although both systems show significantly reduced long-time stabilities under operational conditions, the whole-cell system exhibits one main advantage over the cell-free system. Namely the ability that the catalyst can be re-used for several biotransformation reactions without any loss-of-activity. The observed improved storage stability and rather similar operation stability compared to the cell-free system make the whole-cell catalyst better applicable for industrial processes than the cell-free system. Explanations for the faster inactivation of the cell-free system under storage conditions can be found in the temperature-dependent inactivation of the GlyDH over time. In former studies, the purified GlyDH showed a loss of 60% of its activity over a period of 48 h at 30°C (Richter et al., 2009). Comparing these results to the 80% loss-of-activity using the crude extract in this study under same conditions, the results clearly show that in a cell-free system the GlyDH is responsible for loss-of-activity over time. Regarding the operational stability of both enzyme preparations, the aldehyde, especially the investigated glyceraldehyde, might have a negative effect on both enzyme activity and permeabilization of the cells. These effects combined are potentially the reason why both preparations show comparable inactivation profiles under conditions where glyceraldehyde is converted to glycerol.

Regarding the use of external cofactor we could show that the reaction rate could be improved by factor 4.6 by the addition of a low $NADP^+$ concentration of 1 mM. Due to the fact that intact cells are not able to take up externally added cofactor efficiently, this effect can be explained by a slight permeabilization of the cells through the freezing procedure while storing the catalyst at −20°C. This kind of permeabilization causes a decrease of the internal cofactor level which leads to reduced enzyme activities, which can be counterbalanced by the addition of cofactor. This statement was underlined by the observed enhancement in the reaction rate when cofactor was externally added.

But even if the reaction rates are improved by the addition of external cofactor the conversion still proceeds in absence of externally added cofactor, indicating that the amount of intracellular cofactor is sufficient for a preparative conversion (Gröger et al., 2006a,b; Menzel et al., 2004). Therefore, in terms of applicability one has to decide whether to use high catalyst concentrations and longer incubation periods without cofactor addition or lower catalyst concentrations and shorter reaction times with cofactor addition.

Since pH and temperature are also crucial parameters, the dependence of the catalyst on pH and temperature has been investigated to perform conversions under optimal conditions.

A whole-cell system has many advantages compared to the cell-free system. Gröger et al. have compared the economy of isolated enzymes and whole-cells. Due to the more extensive procedures and the need of adding expensive cofactor to the isolated enzymes, the biotransformation with whole-cells is a cost-attractive alternative (Gröger et al., 2006b). Since GlyDH showed excellent enantioselectivity in the reduction of racemic glyceraldehyde (Richter et al., 2009), our primary focus was to transfer the kinetic resolution of racemic glyceraldehyde to the whole-cell system. There are some kinetic resolutions with oxidoreductases in whole wild-type cells published (Buisson et al., 2000; Eh and Kalesse, 1995; Hioki et al., 2000; Stewart, 2001), whereas the use of a recombinant whole-cell system is not so common (Kataoka et al., 2008). Fortunately, the use of the whole-cell system was possible catalysing the kinetic resolution of racemic glyceraldehyde with an EE of 98% and a conversion of 54% on preparative scale (4 g). Compared to the process using the cell-free system selectivity and conversion are in a similar range. But in terms of stability, recycling, and addition of external cofactor, the whole-cell process is significantly better than the cell-free process.

In conclusion, we could demonstrate that the constructed whole-cell catalyst makes the valuable chiral building block L-glyceraldehyde accessible in a direct enzymatic one-step transformation, even without the need of adding expensive cofactor.

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References

Andrews GC, Crawford TC, Bacon BE. 1981. Stereoselective, catalytic reduction of L-ascorbic-acid—A convenient synthesis of L-gulono-1,4-lactone. J Org Chem 46:2976–2977.

Areces P, Carrasco E, Light ME, Santos M, Plumet J. 2007. Synthesis of enantiopure 4-(dihydroxyalkyl)-β-lactams from D-mannitol using Ley's BDA-protected L-glyceraldehyde. Synlett 3180–3182.

Baer E, Fischer HOL. 1939. Studies on acetone-glyceraldehyde IV. Preparation of d(+)-acetone glycerol. J Biol Chem 128:463–473.

Bertani G. 1951. Studies on lysogenesis: 1. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293–300.

Bi J, Aggarwal VK. 2008. Application of furyl-stabilized sulfurylides to a concise synthesis of 8a-epi-swainsonine. Chem Commun 120–122.

Bommarius AS, Schwarm M, Drauz K. 1998. Biocatalysis to amino acid-based chiral pharmaceuticals—Examples and perspectives. J Mol Catal B-Enzyme 5:1–11.

Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254.

Breuer M, Ditrich K, Habicher T, Hauer B, Keßeler M, Stürmer R, Zelinski T. 2004. Industrial methods for the production of optically active intermediates. Angew Chem-Int Edit 43:788–824.

Buisson D, Baucherel X, Levoirier E, Juge S. 2000. Baker's yeast reduction of α-alkyl-α-hydroxy-β-keto esters. Tetrahedron Lett 41:1389–1392.

Bull JA, Balskus EP, Horan RAJ, Langner M, Ley SV. 2007. Total synthesis of potent antifungal marine bisoxazole natural products bengazoles A and B. Chem Eur J 13:5515–5538.

Chenault HK, Whitesides GM. 1987. Regeneration of nicotinamide cofactors for use in organic-synthesis. Appl Biochem Biotechnol 14:147–197.

Crocq V, Masson C, Winter J, Richard C, Lemaitre Q, Lenay J, Vivat M, Buendia J, Prat D. 1997. Synthesis of trimegestone: The first industrial application of bakers' yeast mediated reduction of a ketone. Org Process Res Dev 1:2–13.

Eh M, Kalesse M. 1995. Remarkable kinetic resolution of chiral β-keto-esters by bakers-yeast reduction. Synlett 837–838.

Ernst M, Kaup B, Müller M, Bringer-Meyer S, Sahm H. 2005. Enantioselective reduction of carbonyl compounds by whole-cell biotransformation, combining a formate dehydrogenase and a (*R*)-specific alcohol dehydrogenase. Appl Microbiol Biotechnol 66:629–634.

Fujita Y, Ramaley R, Freese E. 1977. Location and properties of glucose dehydrogenase in sporulating cells and spores of *Bacillus subtilis*. J Bacteriol 132:282–293.

Galkin A, Kulakova L, Yoshimura T, Soda K, Esaki N. 1997. Synthesis of optically active amino acids from α-keto acids with *Escherichia coli* cells expressing heterologous genes. Appl Environ Microbiol 63:4651–4656.

Geueke B, Riebel B, Hummel W. 2003. NADH oxidase from *Lactobacillus brevis*: A new catalyst for the regeneration of NAD. Enzyme Microb Technol 32:205–211.

Ghisalba O, Meyer HP, Wohlgemuth R. 2009. Industrial biotransformation. In: Flickinger MC, editor. Encyclopedia of industrial biotechnology. Hoboken, NJ: Wiley (in press).

Goldberg K, Schroer K, Lütz S, Liese A. 2007. Biocatalytic ketone reduction—A powerful tool for the production of chiral alcohols—Part I: Processes with isolated enzymes. Appl Microbiol Biotechnol 76:237–248.

Gröger H, Hummel W, Rollmann C, Chamouleau F, Hüsken H, Werner H, Wunderlich C, Abokitse K, Drauz K, Buchholz S. 2004. Preparative asymmetric reduction of ketones in a biphasic medium with an (*S*)-alcohol dehydrogenase under in situ-cofactor-recycling with a formate dehydrogenase. Tetrahedron 60:633–640.

Gröger H, Chamouleau F, Orologas N, Rollmann C, Drauz K, Hummel W, Weckbecker A, May O. 2006a. Enantioselective reduction of ketones with "Designer cells" at high substrate concentrations: Highly efficient access to functionalized optically active alcohols. Angew Chem-Int Edit 45:5677–5681.

Gröger H, May O, Werner H, Menzel A, Altenbuchner J. 2006b. A "second-generation process" for the synthesis of L-neopentylglycine: Asymmetric reductive amination using a recombinant whole cell catalyst. Org Process Res Dev 10:666–669.

Hanson RL, Schwinden MD, Banerjee A, Brzozowski DB, Chen BC, Patel BP, McNamee CG, Kodersha GA, Kronenthal DR, Patel RN, Szarka LJ. 1999. Enzymatic synthesis of L-6-hydroxynorleucine. Bioorg Med Chem 7:2247–2252.

Hilt W, Pfleiderer G, Fortnagel P. 1991. Glucose dehydrogenase from *Bacillus subtilis* expressed in *Escherichia coli*. 1: Purification, characterization and comparison with glucose dehydrogenase from *Bacillus megaterium*. Biochim Biophys Acta 1076:298–304.

Hioki H, Hashimoto T, Kodama M. 2000. Efficient kinetic resolution of (+/−)-4-methyl-Hajos-Parrish ketone by Baker's yeast reduction. Tetrahedron-Asymmetry 11:829–834.

Hubschwerlen C. 1986. A convenient synthesis of L-(*S*)-glyceraldehyde acetonide from L-ascorbic-acid. Synthesis 962–964.

Hubschwerlen C, Specklin JL, Higelin J. 1995. L-(*S*)-Glyceraldehyde acetonide-(1,3-dioxolane-4-carboxaldehyde, 2,2-dimethyl-, (*S*)-). Org Synth 72:1–5.

Hummel W. 1999. Large-scale applications of NAD(P)-dependent oxidoreductases: Recent developments. Trends Biotechnol 17:487–492.

Jurczak J, Pikul S, Bauer T. 1986. (R)-2,3-O-isopropylideneglyceraldehyde and (S)-2,3-O-isopropylideneglyceraldehyde in stereoselective organic-synthesis. Tetrahedron 42:447–488.

Kataoka M, Yamamoto K, Kawabata H, Wada M, Kita K, Yanase H, Shimizu S. 1999. Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by *Escherichia coli* transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes. Appl Microbiol Biotechnol 51:486–490.

Kataoka M, Ishige T, Urano N, Nakamura Y, Sakuradani E, Fukui S, Kita S, Sakamoto K, Shimizu S. 2008. Cloning and expression of the L-1-amino-2-propanol dehydrogenase gene from *Rhodococcus erythropolis*, and its application to double chiral compound production. Appl Microbiol Biotechnol 80:597–604.

Kosjek B, Nti-Gyabaah J, Telari K, Dunne L, Moore JC. 2008. Preparative asymmetric synthesis of 4,4-dimethoxytetrahydro-2H-pyran-3-ol with a ketone reductase and in situ cofactor recycling using glucose dehydrogenase. Org Process Res Dev 12:584–588.

Kumar I, Bhide SR, Rode CV. 2007. Asymmetric synthesis of a 3,4-substituted pyrrolidine by L-proline catalyzed direct enolexo aldolization. Tetrahedron-Asymmetry 18:1210–1216.

Liese A, Zelinski T, Kula MR, Kierkels H, Karutz M, Kragl U, Wandrey C. 1998. A novel reactor concept for the enzymatic reduction of poorly soluble ketones. J Mol Catal B-Enzyme 4:91–99.

Lilius EM, Multanen VM, Toivonen V. 1979. Quantitative extraction and estimation of intracellular nicotinamide nucleotides of *Escherichia coli*. Anal Biochem 99:22–27.

Menzel A, Werner H, Altenbuchner J, Gröger H. 2004. From enzymes to "designer bugs" in reductive amination: A new process for the synthesis of L-*tert*-leucine using a whole cell-catalyst. Eng Life Sci 4: 573–576.

Moore JC, Pollard DJ, Kosjek B, Devine PN. 2007. Advances in the enzymatic reduction of ketones. Acc Chem Res 40:1412–1419.

Patel RN. 2006. Biocatalysis: Synthesis of chiral intermediates for drugs. Curr Opin Drug Discov Dev 9:741–764.

Patel RN, Goswami A, Chu L, Donovan MJ, Nanduri V, Goldberg S, Johnston R, Siva PJ, Nielsen B, Fan JY, et al. 2004. Enantioselective microbial reduction of substituted acetophenones. Tetrahedron-Asymmetry 15:1247–1258.

Pesti JA, DiCosimo R. 2003. Recent progress in enzymatic resolution and desymmetrization of pharmaceuticals and their intermediates. Curr Opin Drug Discov Dev 6:884–901.

Pollard D, Truppo M, Pollard J, Chen CY, Moore J. 2006. Effective synthesis of (*S*)-3,5-bistrifluoromethylphenyl ethanol by asymmetric enzymatic reduction. Tetrahedron-Asymmetry 17:554–559.

Richter N, Neumann M, Liese A, Wohlgemuth R, Eggert T, Hummel W. 2009. Characterisation of a recombinant NADP-dependent glycerol dehydrogenase from *Gluconobacter oxydans* and its application in the production of L-glyceraldehyde. Chembiochem 10:1888–1896.

Röthig TR, Kulbe KD, Bückmann F, Carrea G. 1990. Continuous coenzyme-dependent stereoselective synthesis of sulcatol by alcohol-dehydrogenase. Biotechnol Lett 12:353–356.

Rouhi AM. 2004. Chiral chemistry. Chem Eng News 82:47–62.

Schmid CR, Bryant JD. 1995. D-(*R*)-Glyceraldehyde acetonide-(1,3-dioxolane-4-carboxaldehyde, 2,2-dimethyl-, (*R*)-). Org Synth 72:6–13.

Schroer K, Mackfeld U, Tana IAW, Wandrey C, Heuser F, Bringer-Meyer S, Weckbecker A, Hummel W, Daußmann T, Pfaller R, et al. 2007. Continuous asymmetric ketone reduction processes with recombinant *Escherichia coli*. J Biotechnol 132:438–444.

Schubert T, Hummel W, Kula MR, Müller M. 2001. Enantioselective synthesis of both enantiomers of various propargylic alcohols by use of two oxidoreductases. Eur J Org Chem 4181–4187.

Stewart JD. 2001. Dehydrogenases and transaminases in asymmetric synthesis. Curr Opin Chem Biol 5:120–129.

Uzura A, Nomoto F, Sakoda A, Nishimoto Y, Kataoka M, Shimizu S. 2009. Stereoselective synthesis of (*R*)-3-quinuclidinol through asymmetric reduction of 3-quinuclidinone with 3-quinuclidinone reductase of *Rhodotorula rubra*. Appl Microbiol Biotechnol 83:617–626.

Vasic-Racki D, Jonas M, Wandrey C, Hummel W, Kula MR. 1989. Continuous (*R*)-mandelic acid production in an enzyme membrane reactor. Appl Microbiol Biotechnol 31:215–222.

Walton AZ, Stewart JD. 2004. Understanding and improving NADPH-dependent reactions by nongrowing *Escherichia coli* cells. Biotechnol Prog 20:403–411.

Weckbecker A. 2005. Entwicklung von Ganzzellbiokatalysatoren zur Synthese von chiralen Alkoholen. PhD Thesis, Heinrich Heine University of Duesseldorf.

Weckbecker A, Hummel W. 2004. Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, $NADP^+$-dependent alcohol dehydrogenase and NAD^+-dependent formate dehydrogenase. Biotechnol Lett 26:1739–1744.

Weckbecker A, Hummel W. 2005. Glucose dehydrogenase for the regeneration of NADPH and NADH. In: Barredo JL, editor. Meth in biotechnology. Totowa, NJ: Humana Press Inc, p. 225–237.

Weckbecker A, Gröger H, Hummel W. 2010. Regeneration of nicotinamide coenzymes: Principles and applications for the synthesis of chiral compounds. Adv Biochem Eng Biotechnol (in press).

Wills M, Hannedouche J. 2002. New methodology for the asymmetric reduction of ketones. Curr Opin Drug Discov Dev 5:881–891.