Biocatalytical production of (5*S*)-hydroxy-2-hexanone

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Biocatalytical approaches have been investigated in order to improve accessibility of the bifunctional chiral building block (5*S*)-hydroxy-2-hexanone (**(*S*)-2**). As a result, a new synthetic route starting from 2,5-hexanedione (**1**) was developed for **(*S*)-2**, which is produced with high enantioselectivity (*ee* >99%). Since **(*S*)-2** can be reduced further to furnish (2*S*,5*S*)-hexanediol (**(2*S*,5*S*)-3**), chemoselectivity is a major issue. Among the tested biocatalysts the whole-cell system *S. cerevisiae* L13 surpasses the bacterial dehydrogenase ADH-T in terms of chemoselectivity. The use of whole-cells of *S. cerevisiae* L13 affords **(*S*)-2** from prochiral **1** with 85% yield, which is 21% more than the value obtained with ADH-T. This is due to the different reaction rates of monoreduction (**1**→**2**) and consecutive reduction (**2**→**3**) of the respective biocatalysts. In order to optimise the performance of the whole-cell-bioreduction **1**→**2** with *S. cerevisiae*, the system was studied in detail, revealing interactions between cell-physiology and xenobiotic substrate and by-products, respectively. This study compares the whole-cell biocatalytic route with the enzymatic route to enantiopure **(*S*)-2** and investigates factors determining performance and outcome of the bioreductions.

Introduction

(5*S*)-Hydroxy-2-hexanone (**(*S*)-2**) is a valuable bifunctional chiral building block for *e.g.* pharmaceuticals or aroma compounds. However its poor availability counts against its broad synthetic use. It is for this reason that a new synthetic route was required that allows access to this hydroxy ketone in high selectivity and enantiopurity. Due to their unsurpassed enantio- and chemoselectivity, biocatalytic approaches lend themselves to this purpose.[1] If prochiral diketones are subjected to reduction, two classes of products are accessible. Monoreduction will yield the corresponding bifunctional hydroxyketones, whereas bis-reduction furnishes the corresponding diols. In the case of the γ-diketone 2,5-hexanedione (**1**) reduction affords 5-hydroxy-2-hexanone (**2**) and 2,5-hexanediol (**3**) (Scheme 1). As enantiopure chiral compounds, both products serve as chiral intermediates. While both enantiomers of **3** are key building blocks of chiral catalysts,[2,3] enantiopure **2** is essential for the synthesis of biodegradable polymers, pharmaceuticals or aroma compounds.[4] Though there is a substantial demand for enantiomers of **2**, previous attempts (both biocatalytic and non-biocatalytic) focused on the production of enantiopure **(2*R*,5*R*)-** and **(2*S*,5*S*)-3**,[2,4–12] thus underlining the challenge of producing enantiopure **2**. Nevertheless, efforts had been made to selectively obtain **2** enantiomerically pure. In the case of **(*R*)-2**, a recently developed route employing whole-cells of *Lactobacillus kefiri* allowed for producing **(*R*)-2** with >99% *ee* and 95% selectivity.[13] However enantiomer **(*S*)-2** is not accessible *via* this route. Moreover in order to produce enantiopure **(*S*)-2**, one has to resort to procedures with unsatisfactory cost-efficiency or lower applicability on a larger scale. Non-biocatalytic variants for production of enantiopure **(*S*)-2**, such as asymmetric catalytic hydrogenation of **1** with Ru-BINAP[4] and oxidation of **(2*S*,5*S*)-3**, have only been successfully applied for production of the respective (*R*)-enantiomers[4,14] and suffer from harsh reaction conditions or inefficient use of highly valuable reactants.

Among the existing biocatalytical approaches to enantiopure hydroxyketones, lipase-catalysed dynamic kinetic resolution of

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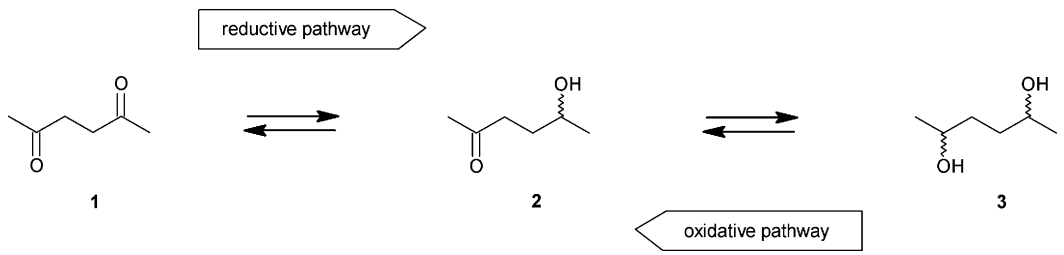


Scheme 1 The prochiral 2,5-hexanedione (**1**) and chiral hexanediol (**3**) are precursors for 5-hydroxy-2-hexanone (**2**); thus **2** can be obtained through either a reductive or an oxidative pathway. Both options are discussed in the text.

***rac*-2** showed poor enantioselectivity (*E*-value = 9 for **(*R*)-2**)[15] and thus is not able to resolve the racemic mixture of ***rac*-2** satisfactorily.

An alternative dehydrogenase-catalysed route with whole-cells of *Rhodococcus ruber* gave only moderate yields (65% conversion, 38% **(*S*)-2**, *ee* >99%).[16] Another approach consists in the oxidation of ***meso*-3**, which furnished **(*R*)-2** in up to 88% yield with >99% *ee*.[17,16] Via a (*R*)-selective reductase, *e.g.* ADH-LK or ADH-LB from *Lactobacillus* sp.,[18] this approach is the only one reported thus far which is applicable for the synthesis of **(*S*)-2** as well. However, the major drawback of this procedure is that the availability of enantiopure ***meso*-3** is not given, and it is neither commercially available nor efficiently producible. Furnishing ***meso*-3** from ***rac*-3** means laborious multi-step syntheses and low product yields not exceeding 21%.[19,20] Consequently, this strategy must be considered not effective, since it involves considerable expense and suffers from poor substrate availability.

Nevertheless, in view of the rather harsh reaction conditions of the transition metal catalysed variants, and in light of the positive results emerging from the oxidative route that showed **(*S*)-2** and ***meso*-3** to be interconvertible by means of dehydrogenase catalysis, we were encouraged to study alternative dehydrogenase-catalysed approaches to efficiently and cost-effectively produce enantiopure **(*S*)-2** from **1**, in order to overcome the synthetic bottleneck in the chemistry of **(*S*)-2**.

Results and discussion

Synthesis of (5*S*)-hydroxy-2-hexanone (**(*S*)-2**) through reduction of prochiral 2,5-hexanedione (**1**) is the most efficient approach, not only in terms of atom economy[21] but also in terms of cost and availability of the starting material. Potentially suitable biocatalysts for this process are preferentially those which have been employed for production of (2*S*,5*S*)-hexanediol (**(2*S*,5*S*)-3**), since the reduction of the two carbonyl groups of **1** proceeds in a consecutive manner, in which an intermediate (in this case **(*S*)-2**) accumulates transiently. Hence, if the biocatalytic reduction of **1** basically obeys the kinetics of a consecutive reaction, a detailed investigation of the process will allow for identifying conditions permitting the isolation of intermediate product **(*S*)-2**.

In this study two biocatalysts were found potentially suitable for the production of **(*S*)-2**: on the one hand the cost-effective and easy-to-use whole-cell-biocatalyst *Saccharomyces cerevisiae* known to selectively reduce **1** to **(2*S*,5*S*)-3**,[5,6] and on the other an isolated dehydrogenase from *Thermoanaerobacter* sp., which has been employed recently to develop an efficient procedure yielding enantiopure **(2*S*,5*S*)-3** through reduction of **1**.[22]

The whole-cell-biocatalyst *Saccharomyces cerevisiae*

Versatility of this whole-cell-biocatalyst has long been recognised,[23–25] but there are no reports in the literature investigating the applicability of this biocatalyst for the production of enantiopure **(*S*)-2**.

One reason for disregarding *S. cerevisiae* as a biocatalyst maybe the diversity of yeast strains in use, differing in activity and selectivity, which in turn complicates the portability of developed protocols. Furthermore, at least 49 open-reading-frames coding for dehydrogenases in the *S. cerevisiae* genome[26] are known, which often have counteracting stereoselectivity and thus are the reason for unsatisfactory enantiopurities encountered in a majority of whole-cell biotransformations with *S. cerevisiae*.[26–28]

However, the latter fact cannot be generalised, since the stereoselectivity of a given bioreduction not only depends on the kind of the reactant but also on the composition of the reductase-pool in the cell, which in turn is adjusted to the current environmental conditions *via* stress-response pathways in order to maintain cellular homeostasis. In this context, cell-stress is commonly referred to environmental conditions that threaten the survival of a cell, or at least prevent it from performing optimally.[29]

But stress responses of whole-cells can also be used to control stereoselectivity of reductions of xenobiotics by adding a physiologically active substance.[30] Thus expression of reductases may be altered without the need for genetic modification. Hence the use of whole-cells of *S. cerevisiae* should not be ruled out *a priori*. In particular, its use offers important advantages like intracellular cofactor regeneration and a very good availability,[31] allowing for easy up-scaling of a procedure. In the end, every reactant has to be evaluated with regard to whether *S. cerevisiae* is a suitable biocatalyst for an intended biotransformation, and whether the mentioned disadvantages outweigh the advantages of this versatile biocatalyst. Consequently, the applicability of the whole-cell-biocatalyst *S. cerevisiae* for production of **(*S*)-2** needs to be investigated.

In order to study a strain with preferably high activity towards **1**, we compared the commonly used laboratory-yeast strains CEN.PK 113–7D (haploid)[32] with the industrial model-strain CBS 8066 (diploid)[33] and the industrially produced strain L13 (polyploid: tetraploid and aneuploid).[34] The diversity in activity towards reduction of **1** among the studied yeast strains is shown in Table 1. The results clearly show that all strains accept **1** as a xenobiotic substrate but that it is advisable to use the industrial strain L13 due to its superior activity. Thus, the intracellular level of 2,5-hexanedione-reductase(s) (in the following abbreviated as HDOR) and hence the activity of the respective whole-cell-biocatalyst varies from strain to strain.

This observation may have its reason in the different ploidy of the strains, which has been found to correlate with protein content of the cells under optimal conditions[35,36] and would thus potentially affect enzyme activity. Furthermore the strains are not isogenic, so expression level or even the structure of the respective HDOR are potentially different,[37] which can result in a different activity of the whole-cell-biocatalyst towards **1**. However extensive elucidation of the reasons for strain-dependent differences in activities of xenobiotica-accepting reductases is too complex to be addressed here in detail. In the light of these results the strain with the highest activity, *S. cerevisiae* L13, was selected for further studies.

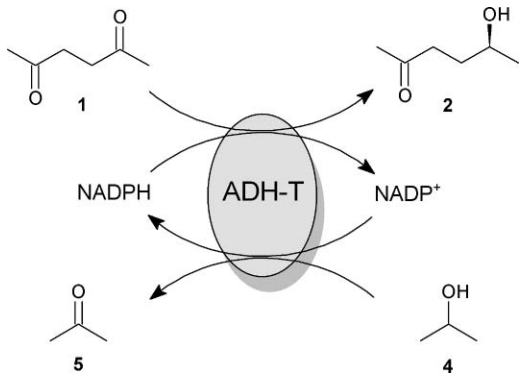
Table 1 2,5-Hexanedione reducing activity varies among yeast strains

Strain	Relative 2,5-hexanedione reducing activity
S. cerevisiae L13	100%
S. cerevisiae CBS 8066	58%
S. cerevisiae CEN.PK 113–7D	61%

Alcohol-dehydrogenase from *Thermoanaerobacter* sp. (ADH-T)

The second promising biocatalyst, investigated in terms of production of **(*S*)-2**, is a (*S*)-selective alcohol-dehydrogenase from a thermophilic bacterium (ADH-T) which has recently been used to develop an efficient procedure to obtain enantiopure **(2*S*,5*S*)-3**.[22] ADH-T is available in an isolated form, which results in advantages like high specific activity towards its substrates and a well-defined system. However since the enzyme depends on the cofactor NADPH, which, due to its price, cannot be used in stoichiometric amounts, care has to be taken on efficient regeneration of the cofactor in order to get a cost-effective system. Often enzyme-coupled cofactor regeneration is employed, which necessitates an additional enzyme together with its corresponding substrate, to reduce $NADP^+$, *e.g.* glucose-dehydrogenase/glucose.[38] In this context an important advantage of ADH-T becomes obvious. Since it is capable of catalysing oxidation of secondary alcohols with a sufficient rate even at pH 7, its pH-optimum for reduction,[39] the use of ADH-T allows for a substrate-coupled cofactor regeneration approach, thus abolishing the need for an additional enzyme.

The use of ADH-T permits the use of 2-propanol (**4**) as a hydrogen donor, which is oxidised to acetone (**5**), regenerating the NADPH needed for reduction of **1** (Scheme 2).



Scheme 2 Substrate-coupled production of **(*S*)-2** employing ADH-T. Cofactor regeneration and reduction of 2,5-hexanedione (**1**) are catalysed by one and the same enzyme.

Due to the laws of mass action, the overall equilibrium of the whole system only depends on the free energies of the two alcohols and the corresponding ketones, in which the equilibrium constant is determined through eqn (1). Hence, if full conversion of **1** is intended the equilibrium can be shifted towards **3** through removal of **3** or acetone as well as the use of **4** in large excess.

$$K_{eq} = \frac{c_{eq}(\mathbf{3}) \cdot c^2_{eq}(\mathbf{5})}{c_{eq}(\mathbf{1}) \cdot c^2_{eq}(\mathbf{4})} \quad (1)$$

However since selective removal of **3** is not easily achieved, it is obvious that full conversion can only be obtained through the use of excess **4** and removal of formed **5**. However if full conversion is not intended, as is the case in the investigation of ADH-T-catalysed formation of **(*S*)-2**, it was found that acetone removal is not necessary in order to reach the maximal concentration of **2**.

Although the maximal cofactor-regenerating capacity of the system in Scheme 2 is not required to study mono-reduction of **1**, it will be of use in a final process in order to improve efficiency by saving 2-propanol.

Bioreduction of 2,5-hexanedione through ADH-T and *S. cerevisiae* L13

With the aforementioned considerations in mind, bioreduction of **1** was achieved by using either ADH-T with substrate-coupled cofactor-regeneration employing 2-propanol or resting whole-cells of *Saccharomyces cerevisiae* L13 (SCL13), which were supplied with sucrose as the sole carbon source. From the representative time-courses of the respective reductions depicted in Fig. 1, it is obvious that both reductions obey the kinetics of a consecutive reduction. That is, intermediate **2** accumulates transiently in the course of the reaction and its maximal concentration is reached under non-equilibrium conditions. Since the desired product **2** forms faster than **3**, it is advisable to control the reaction kinetically rather than thermodynamically (equilibrium-controlled). Investigations into condensation reactions in which the desired product hydrolysed subsequently (and thus can be understood as an intermediate of a consecutive reaction) showed that a higher product yield could be obtained more rapidly in a kinetically controlled process than in an equilibrium controlled process.[40] These observations and considerations show that production of **2**

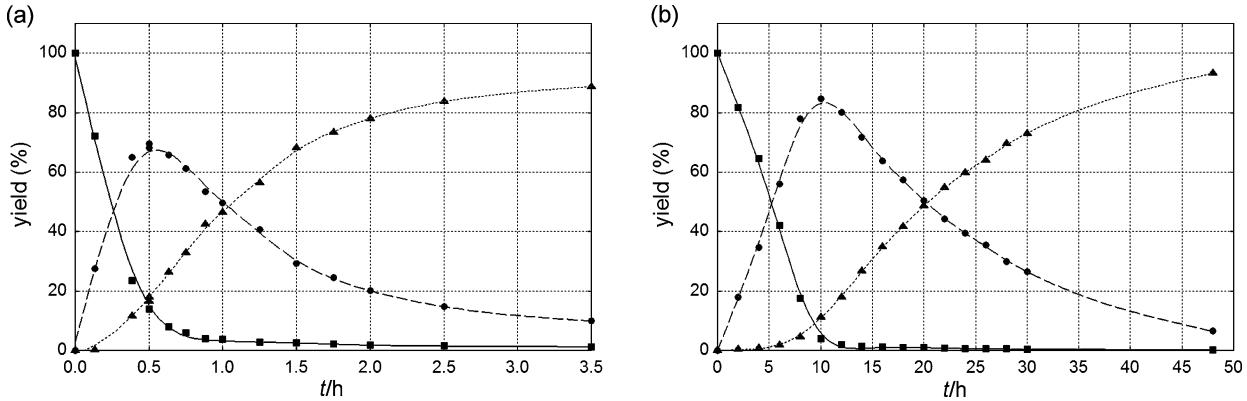


Fig. 1 Time course of a bioreduction of 2,5-hexanedione (■) (80 mM): **(a)** with ADH-T, employing substrate-coupled cofactor regeneration *via* 2-propanol (2 M) without removing acetone at pH 7.0; **(b)** with whole-cells of *S. cerevisiae* L13 (SCL13) supplied with sucrose; both at 30 °C (5-hydroxy-2-hexanone (●); 2,5-hexanediol (▲)).

is possible if the reaction is conducted under kinetic control, *i.e.* the reaction is stopped at maximal concentration of **2**, yielding a reaction medium containing the maximum achievable product concentration of **2** under the prevailing reaction conditions.

Determination of product enantiopurities gave that both reactions (**1**→**2** and **1**→**3**) proceed with very high enantioselectivity yielding both **(*S*)-2** and **(2*S*,5*S*)-3** in >99% *ee*.

This extraordinarily high stereoselectivity indicates that in the case of SCL13 no counteracting reductase reducing **1** with different stereoselectivity is present in the cell, which furthermore underlines that this whole-cell biocatalyst must not be regarded unselective in general.

The observation that conversion of **1**→**2** with ADH-T is achieved in a fraction of time needed by SCL13 has its reason in the concentration of **1**-reducing dehydrogenases in the respective experiment. Application of isolated enzymes like ADH-T allows for higher concentrations of **1**-reducing dehydrogenases since the plethora of enzymes needed to maintain a whole-cell are not present. Thus higher activity per volume unit can be achieved with isolated enzymes. Unfortunately HDOR from SCL13 is not yet available in an isolated form since identification and isolation are part of ongoing studies. However, it has to be stressed here that compared to isolated enzymes, the use of whole-cell-biocatalysts may be less favourable in terms of activity, but this drawback is compensated for by the much lower price and the ease of handling (*e.g.* no care has to be taken on cofactor regeneration) and thus still renders the use of whole-cell biocatalyst economically attractive.

Besides these considerations, the major advantage of using SCL13 is the high peak concentration of **2**, which is 21% higher than that achieved with ADH-T. Thus SCL13 allows for a more efficient synthesis of **(*S*)-2**, since the amount of remaining reactant **1** and secondary product **3** is reduced, which simplifies downstream processing.

Since the system is kinetically controlled, the kinetic parameters of the biocatalyst determine the maximal peak concentration of **2**. This is also reflected by the apparent kinetic parameters determined in the presence of saturating concentrations of cofactor NADPH (Table 2). From these parameters is evident that the affinity of both biocatalysts for **1** is higher than for **2** (K_M(**2**)/K_M(**1**) = 4.1 for both biocatalysts), which basically is advantageous for accumulation of **2**. However, since the difference and thus the ratio K_M(**1**)/K_M(**2**) is comparable for both biocatalysts, K_M cannot account for the better performance of SCL13. Considering the apparent maximal velocities of both reactions (Table 2) it becomes evident that the difference between v_1(**1**→**2**) and v_2(**2**→**3**) is much more pronounced for HDOR of SCL13 than for ADH-T (v_1(**1**→**2**)/v_2(**2**→**3**): SCL13 = 15.3; ADH-T = 1.8), which results in the superior chemoselectivity of SCL13 in production of **2**. Given that both reactions are reversible in general, oxidation of **2** and **3** was also investigated.

Table 2 Kinetic constants determined for HDOR and ADH-T

	Reaction	K_M[b]	v_{max}[b,c]
S. cerevisiae L13[a]	**1**→**2**	2.5 mM	94.7 mU/mg
	2→**3**	10.2 mM	6.2 mU/mg
	3→**2**	42.8 mM	23.8 mU/mg
	2→**1**	3.4 mM	28.0 mU/mg
ADH-T[a]	**1**→**2**	0.2 mM	27.3 kU/mg
	2→**3**	0.7 mM	15.0 kU/mg
	3→**2**	1.5 mM	15.8 kU/mg
	2→**1**	0.2 mM	3.6 kU/mg

[a] For experimental conditions see experimental section. [b] Apparent values under saturating conditions of cofactor. [c] No k_{cat} given since biocatalysts were not purified to homogeneity.

In the presence of saturating concentrations of $NADP^+$, oxidation of **3** with HDOR of SCL13 will only become effective at a high concentration of **3** due to its high K_M (42.8 mM), whereas the kinetic parameters of ADH-T (K_M(**3**) = 1.5 mM) for oxidation of **3** allow for becoming effective at lower concentrations of **3**. However, the subsequent oxidation of **2** through ADH-T will be slower when compared to oxidation of **3** because of the slower apparent maximal velocity (Table 2). Hence oxidation of **3** through ADH-T will also lead to an accumulation of **2**, which is not the case if **3** is oxidised by HDOR of SCL13, because this biocatalyst would catalyse oxidation of **2** at a higher rate, due to K_M(**2**) < K_M(**3**) (Table 2), if $c < 10K_M$. However, oxidation of substrates **2** and **3** will play a minor role if both biocatalysts are used to produce **2** by reduction of **1**, a process that is more effective than the oxidation of highly valuable enantiopure **(2*S*,5*S*)-3**. Under these conditions reduction of **1** will be favoured through an excess of NADPH over $NADP^+$ in the cells, which was found in a number of yeast strains.[33,41] In the case of a system employing ADH-T, oxidation of alcohols is the key to regenerate $NADP^+$. However, the excess of 2-propanol will competitively inhibit oxidation of **2** and **3**, respectively, although the contribution of oxidation of **3** to accumulation of **2** cannot be neglected and thus contributes to accumulation of **2**.

A full characterisation of both biocatalysts in detail requires the pure enzymes for which the assumed ordered bi–bi mechanism has to be validated, followed by determination of the 16 individual rate constants as defined in the rate equation,[42] and thus goes far beyond the scope of this contribution.

Although the kinetic parameters of the biocatalyst determine the maximal yield of **2** and hence the chemoselectivity of the bioprocess, it is also essential to study factors affecting the activity of the whole-cell-biocatalyst in order to identify conditions allowing for its most efficient use.

Factors affecting the activity of 2,5-hexanedione-reductases (HDOR) in *S. cerevisiae* L13

Besides strain-dependent variation, the physiological status of the respective whole cell biocatalyst is a potential affector of HDOR-activity. As the expression of dehydrogenases is altered through a number of stress-responses in *S. cerevisiae*,[43–45] expression of HDOR is most likely affected during exposure of yeast cells to stress. Hence, conditions should exist under which the activity of the whole-cell-biocatalyst is improved through stress-exposure. As is evident from Fig. 2a, exposing exponentially growing cells to heat shock, osmotic stress or chemical stress increases the activity of HDOR. Furthermore, Fig. 2a also shows that HDOR-activity resulting from exposure of *S. cerevisiae* L13 to osmotic stress by far exceeds activities resulting from exposure to other conditions.

The induction of HDOR had no effect on stereoselectivity of the biocatalyst, which underlines that no dehydrogenases accepting **1** as a substrate with counteracting selectivity were induced through the provoked stress responses.

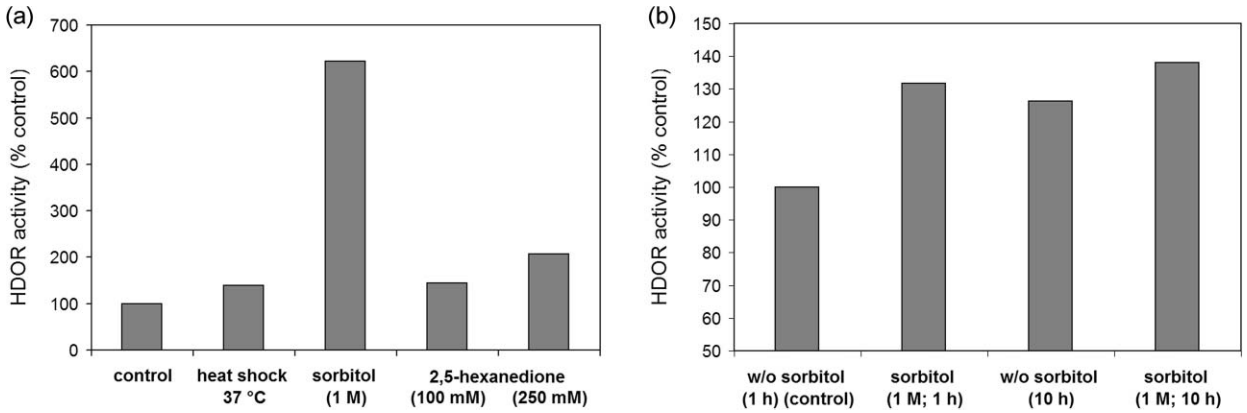


Fig. 2 **(a)** Induction of HDOR in exponentially growing *S. cerevisiae* L13 (SCL13) after exposure to heat-shock (37 °C), osmotic-stress (1 M sorbitol) and chemical-stress (2,5-hexanedione) for 1 h. **(b)** Induction of HDOR-activity in resting *S. cerevisiae* L13 under conditions of a biotransformation and exposure to osmotic stress.

Comparison of the activity of exponentially growing cells with that of commercial grade SCL13 (compressed yeast) revealed that the latter exhibit an up to fourfold higher HDOR-activity (76 mU/mg(protein) vs. 19 mU/mg(protein) in exponentially growing cells). This observation is reasonable since industrially produced compressed yeast cells are exposed to a lack of nutrients at the end of the production process, which constitutes a stressful condition, employed in order to increase robustness of the organisms and shelf-life of the product.[46,47] Hence the increased HDOR-activity of industrial grade SCL13 can be attributed to a stress-response during production and thus underlines the inducibility of HDOR through cell-stress.

The induction of HDOR through multiple stresses suggests that the gene encoding for this enzyme is regulated as for the so-called environmental-stress-response genes, a group of genes whose expression is altered by diverse types of stress.[48,49] In this group only 9 genes encoding for biotransformation-relevant putative and confirmed dehydrogenases can be found, and will be good candidates in future investigations concerning identification of HDOR.

So exposing cells to stressful conditions prior to or during the biotransformation is of benefit in terms of HDOR-activity. Due to the fact that commercial grade compressed yeast already has a high basic level of HDOR-activity, its use is more practicable than exposing exponentially growing cells to osmotic shock.

Since biotransformations with commercial grade SCL13 are carried out at high cell-densities under non-growing conditions (resting cells) and nitrogen limitation, it remains to be elucidated whether exposure of non-growing cells to osmotic shock under conditions of a biotransformation will also increase HDOR-activity. Fig. 2b shows the results of these experiments. Though induction of HDOR after 1 h and 10 h of exposure to osmotic stress took place, the relative increase of HDOR-activity, related to the control, is much smaller than that observed in exponentially growing cells. This is attributable to the significantly decreased protein biosynthesis in resting, stationary-phase-like cells[50] and the circumstance that expression of every chromosomal encoded protein in a living organism cannot be increased indefinitely, *i.e.* if a maximal HDOR-activity in wild-type yeast-cells is already present, it cannot be increased any further through alteration of biotransformation conditions.

Though osmotic stress induces HDOR in non-growing *S. cerevisiae* L13, it must be noted that prolonged incubation of these cells under biotransformation conditions resulted in a similar increase in HDOR-activity without sorbitol being present (Fig. 2b). Hence the conclusion needs to be drawn that there is no further benefit in applying stressful conditions (for instance by additives in whole-cell biotransformations of **1**), since conditions of the biotransformation itself lead to an increase in the already high activity of HDOR in commercial grade *S. cerevisiae* L13. Though the activity of exponentially growing cells exposed to 1 M sorbitol (up to 130 mU/mg(protein)) was not reached with industrially produced compressed *S. cerevisiae* L13 (up to 100 mU/mg(protein) after incubation under conditions of biotransformation), use of the latter is more practical in terms of price and availability.

After having identified conditions allowing for a maximal HDOR-activity, a comprehensive study of the intended process should not ignore the limitations of the process. Those limitations may arise from the complexity of a whole-cell biocatalyst and the effects of a xenobiotic compound on cell physiology and the metabolic network. Together with side-reactions, these processes may impair the performance of the bioprocess, and thus need to be investigated in order to assess the potential of the process and to identify optimisation strategies.

Limitations of biocatalytic production of (5*S*)-hydroxy-2-hexanone with *S. cerevisiae* L13

Since the outcome of a bioreduction with whole-cells not only depends on the functionality of the dehydrogenase catalysing the reduction, but on the whole cellular metabolic network and the integrity of the cell, vitality of the cells is a major issue.

Effects on cell-vitality. **1** does not affect cell-vitality up to a concentration of 5% v/v (440 mM). Additionally 3-methylcyclopentenone (**7**), a reactant-impurity resulting from intramolecular condensation of **1** and a potential fungicide,[51] will not impair cell-vitality if **1** is used in technical grade (purity >95%) at least. Thus even in batch-biotransformations with 5% **1**, the

concentration of **7** will, in the worst case, not exceed 0.25%, a concentration which has been found to be tolerated by *S. cerevisiae* L13. Moreover, *S. cerevisiae* L13 is capable of reducing **7**, forming 3-methylcyclopentanone (**8**).

Formation of by-products and interaction with cell physiology. Though vitality of *S. cerevisiae* is not significantly impaired even at high concentrations of **1**, metabolic processes are affected at lower concentrations. This has its reason in the electrophilic properties of **1**, making it capable of reacting with nucleophiles like protein amino- or thiol-groups. Whereas reaction with the former, furnishes (semi)-thioacetals (**16**) the latter reaction gives rise to the formation of Schiff-bases (**13**, **15** and **9**) and pyrroles (**14**), respectively (Scheme 3), resulting in the modification of proteins through **1** and **2**. Furthermore **1** is also capable of crosslinking proteins through pyrroles or reaction with two amino-groups from different proteins (**13**→**15**).

Since thiol- and amino-groups are often essential for catalytic activity of enzymes,[52,53] modification of such residues would inhibit the activity of the effected enzyme. Indeed, inhibition of the key glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase was found in *S. cerevisiae* L13 exposed to **1** (Fig. 3), which is corroborated by studies with rats in which **1** was found to affect the activity of phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase.[54,55] However, inhibition of glyceraldehyde-3-phosphate dehydrogenase in *S. cerevisiae* is not fatal for cellular metabolism, since glycolytic enzymes are present in high abundance.[56] However, further investigations showed that **1** also affects the activity of the lower abundant enzyme glucose-6-phosphate dehydrogenase (Zwf1p) (Fig. 3),

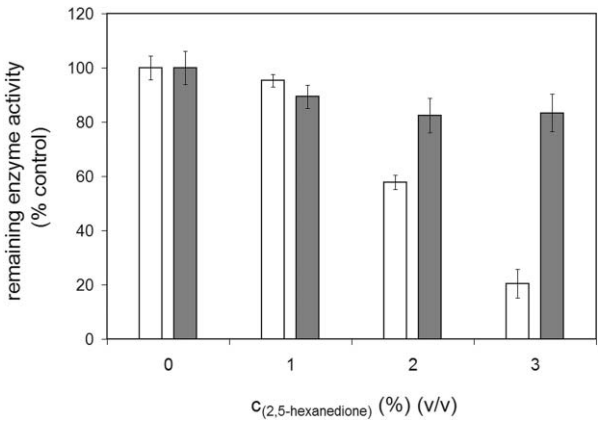
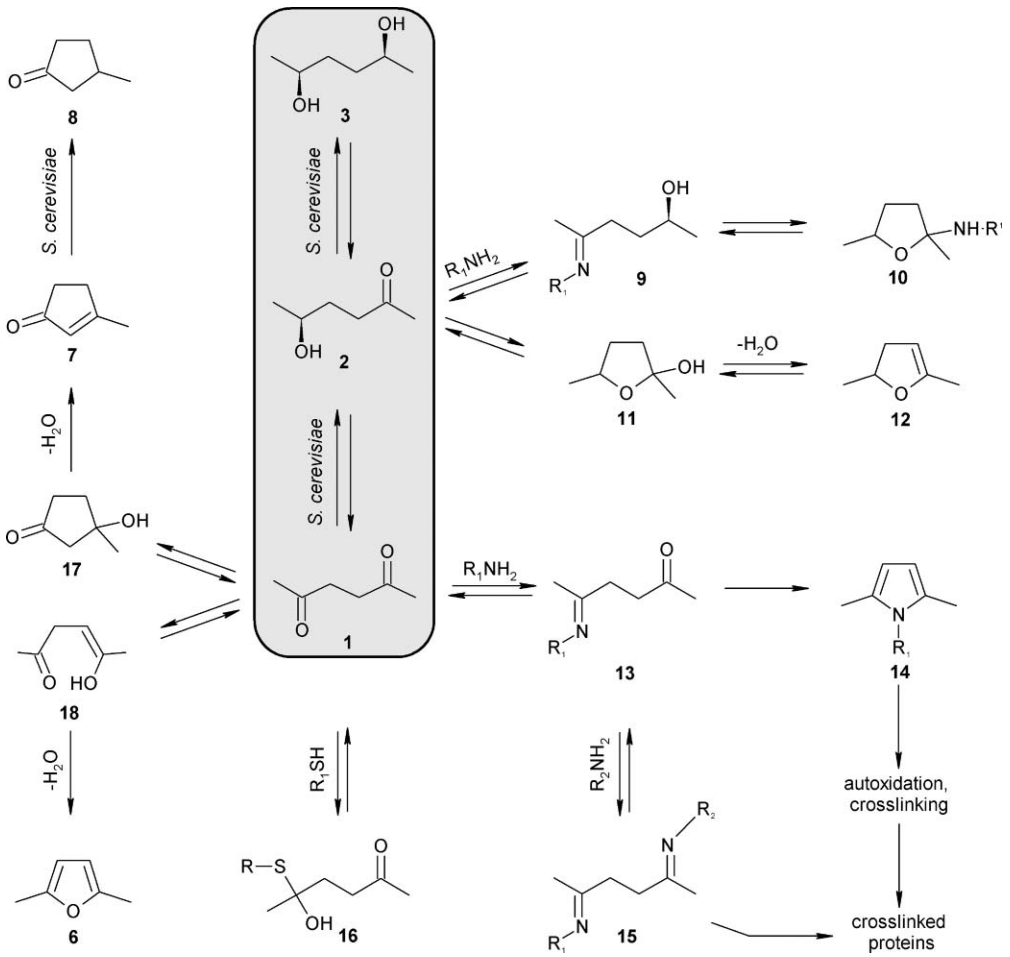


Fig. 3 Remaining activity of glyceraldehydes-3-phosphate dehydrogenase (grey bars; 100% correspond to 10.9 U/mg(protein)) and glucose-6-phosphate dehydrogenase (white bars; 100% correspond to 0.4 U/mg(protein)) in *S. cerervisiae* L13 after exposure to different concentrations of 2,5-hexanedione after 24 h under biotransformation conditions.



Scheme 3 Overview of side reactions in biotransformations of 2,5-hexanedione.

which is especially important in biotransformations, since it is the major NADPH-regenerating enzyme in *S. cerevisiae*.[57]

The reduced activity of Zwf1p after 24 h of bioreduction of **1** has to be attributed to inhibition of this enzyme through **1** since its expression is reported to be essentially constitutive.[58] Thus it can be ruled out that this significant decrease in activity of Zwf1p is due to repression through stress-responses provoked by **1**. Inhibition of glucose-6-phosphate dehydrogenase may result in a decrease in the velocity of reduction of the prochiral ketones **1** and **2**, which depends on NADPH.

Furthermore, NADPH is required to detoxify reactive oxygen species (ROS) and needed to maintain the level of reduced glutathione, the key determinant of intracellular redox-status.[59,60] Thus the probability of oxidative damage and oxidative stress increases with decreasing intracellular NADPH-levels.

Formation of by-products throughout the bioreduction not only affects cell physiology but also product yield, since competing reactions consume reactant and product, respectively. The investigated side-reactions were found not to significantly impair product yield and can be assessed to account for a maximal loss of around 1% of starting material.

Much more important is the ability of **2** to undergo intramolecular cyclisation with subsequent dehydration, furnishing 2,3-dihydro-2,5-dimethylfuran. Through this reaction pathway, **2** would be continuously withdrawn from the reaction, thus impairing product yield. Fortunately, acetalisation of **2** is an equilibrium-dependent process[61,62] in which **2** mainly exists in the open chain form as long as water is present.[63–66] This observation can be ascribed to the limited number of stabilising intramolecular hydrogen bonds in hemiacetal **11**. When compared to the most prominent 2-keto-alcohol: D-fructose, it is obvious that the β-D-fructofuranose has more possibilities to stabilise *via* intramolecular hydrogen bonds (Scheme 4) than **11**, and hence it is reasonable that D-fructose exists mainly in the hemiacetal form, whereas **2** mainly exists in the open-chain-conformation.

11 β-D-fructofuranose

Scheme 4 A network of intramolecular hydrogen bonds (possible H-bonds are represented by dashed lines) stabilises the hemiacetal of β-D-fructose. Hemiacetal **11** cannot stabilise in the same manner due to the lack of capability to form an intramolecular hydrogen bond network. Data on hydrogen bonds for β-D-fructofuranose is reproduced from quantum mechanics/molecular mechanics studies.[73]

Hence the possibility of product loss through formation of 2,3-dihydro-2,5-dimethylfuran during the biotransformation in water can be neglected. The situation becomes different if the product is freed from water due to purification or storage as a neat substance. Under these conditions cyclisation and dehydration are favoured, especially if metal ions, catalysing the reaction, are present. Dihydrofurans easily auto-oxidise and crosslink and thus contaminate the product.[67,65] Hence it is advisable to store **2** as an aqueous solution or carry out subsequent reactions in one pot together with the biotransformation. If **2** is needed as a neat substance, substantial losses (>50%) in isolated yield have to be tolerated.

Adsorption and absorption through yeast. Another limiting issue observed is the capability of yeast cells to bind a proportion of reactant and product, either by absorption and retention in the cells' cytoplasm or adsorption on the outer cell wall. Though this behaviour is of benefit in order to remove (for instance) mycotoxins,[68] it leads to an apparent lower substrate and product concentration in the supernatant of a bioreduction after removal of biomass, and necessitates extraction of the cell pellet if a maximal product yield is desired. After 48 h of bioreduction of **1** (batch with 1% v/v **1**) 20% of the resulting mixture of **1**, **2** and **3** were found to be adsorbed and absorbed by the employed yeast cells. Since adsorption and absorption of substrates and products is also described for ethyl 3-oxobutanoate (20–30% adsorbed and absorbed after 24 h),[31] in general care has to be taken to properly extract the cells during down-stream processing in order to avoid significant product loss.

Cell-free bioreduction

In order to by-pass the aforementioned drawbacks, cell-free approaches could provide a solution. The feasibility of reduction of **1** in a cell-free system using yeast dehydrogenases can be exemplified by employing a crude-extract of *S. cerevisiae* L13 with regeneration of cofactor NADPH through glucose-dehydrogenase (Fig. 4). By using this system an even higher peak concentration of **(*S*)-2** could be observed (89%). However, the advantages of a cell-free system are accompanied by the disadvantageous demand for an additional cofactor-regenerating enzyme, which results in increased production costs.

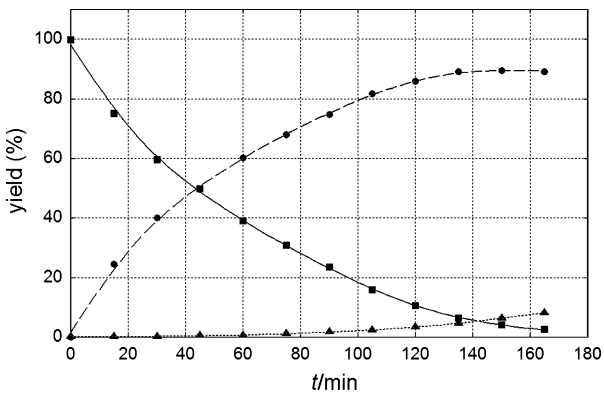


Fig. 4 Representative time course of a bioreduction employing *S. cerevisiae* L13 crude extract (0.8 U/mL 2,5-hexanedione-reductase; 4.5 U/mL glucose-dehydrogenase; 120 mM glucose; 50 mM 2,5-hexanedione; 1 mM $NADP^+$).

The productivity of the suggested system could be increased further by isolation, identification and heterologous expression of the reductase responsible for reduction of 2,5-hexanedione in *S. cerevisiae*. These investigations are currently being examined, and are beyond the scope of this contribution.

Further improvement would aim at increasing the selectivity of the biocatalyst by increasing the ratio of the apparent reaction velocities $v_1(\mathbf{1}\rightarrow\mathbf{2})/v_2(\mathbf{2}\rightarrow\mathbf{3})$, which determines selectivity and yield of **2**.

One possibility is conducting the reduction with $c_0(\mathbf{1}) < K_M(\mathbf{2})$; however, $K_M(\mathbf{1})$ and $K_M(\mathbf{2})$ are both in the mM range and thus would limit the concentration of the product to an inefficient level. Improving $c_{max}(\mathbf{2})$ through reaction engineering is quite challenging since only the second part of the reaction should be affected. However, both reductions (**1**→**2** and **2**→**3**) are catalysed by the same enzyme and hence any change of reaction parameters will always affect both parts of the reaction. Furthermore, a simplified *in silico* model (implemented in COPASI[69]) of the consecutive reaction (**1**→**2**→**3**) obeying reversible Michaelis–Menten kinetics predicts that $c_{max}(\mathbf{2})$ for the intermediate only approaches 100% as the ratio of the reaction velocities v_1:v_2 approaches infinity (Fig. 5). Hence if $c_{max}(\mathbf{2})$ is already high, as is the case in bioreductions of **1** employing *S. cerevisiae* L13, an increase in the difference between v_1 and v_2 will have less effect on $c_{max}(\mathbf{2})$.

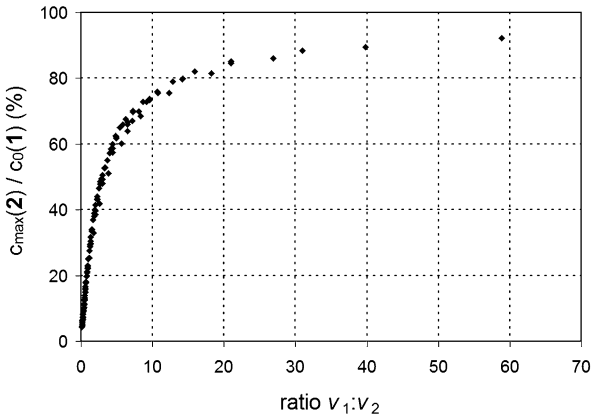


Fig. 5 Prediction of $c_{max}(\mathbf{2})$ in dependence of the ratio of the maximal reaction velocities v_1:v_2. Simulation was carried out with COPASI using a simplified model employing parameters from Table 2 assuming reversible Michaelis–Menten kinetics for the consecutive reduction of **1**.

Another option to increase the yield of **2** is its selective removal from the reaction medium; however, this is quite difficult to achieve since reactant and product are highly similar. A promising alternative would be directed evolution of the enzyme with the aim of decreasing the affinity of the biocatalysts towards **2**, without affecting affinity for **1** and reaction rate for its reduction.

Conclusions

Application of enantiopure hydroxyketones, which are valuable building blocks in organic synthesis, is limited by their poor availability. Hence there is a need to develop strategies for a sustainable and effective production of these building blocks. In this field biocatalytic approaches are most promising due to their unsurpassed selectivity and operation at ambient conditions. In terms of atom economy, the optimal reactant to obtain (5*S*)-hydroxy-2-hexanone (**(*S*)-2**) is the commercially available diketone 2,5-hexanedione (**1**). Since the reduction proceeds as a consecutive reaction finally yielding (2*S*,5*S*)-hexanediol (**(2*S*,5*S*)-3**), the desired product is an intermediate, which transiently accumulates. Comparison of two biocatalysts already successfully applied in enantioselective synthesis of **(2*S*,5*S*)-3** revealed that 2,5-hexanedione-reductase(s) (HDOR) from *S. cerevisiae* L13 are particularly suitable for the production of enantiopure **(*S*)-2** since the developed procedure yields up to 89% **(*S*)-2** from **1** with high stereoselectivity (*ee* >99%).

Further investigations outlined the potentials and limitations of using whole-cells and cell-free approaches. Whereas production of **(*S*)-2** by employing whole-cells of *S. cerevisiae* L13 is cost-effective, limitations arise from interactions of the reactants with cell physiology. In order to by-pass these limitations, cell-free approaches can be used, although these will be more expensive. However, one has the freedom to select the approach towards **(*S*)-2** that best meets the required demands.

Taken together, these studies illustrate the possibilities to produce enantiopure (5*S*)-hydroxy-2-hexanone with either whole-cells of *S. cerevisiae* or a cell-free approach for the first time, and thus improves availability of a versatile chiral building block. Furthermore, the detailed investigation of the processes taking place during the bioreduction contributes to a deeper understanding and allows for the assessment of potentials and optimisation strategies.

Experimental

Chemicals

2,5-Hexanedione (97%) was obtained from Wacker AG and purified to 99% by distillation before use. Chemicals and enzymes used in enzymatic assays were purchased from Sigma. ADH-T and glucose dehydrogenase (GDH) was kindly provided by Julich Chiral Solutions GmbH, a Codexis company. All other chemicals were obtained from Fluka and Acros. Sucrose was obtained from a local store.

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were: CBS8066 (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands); L13 (FALA Societé industrielle de levure, Strasbourg, France) and CEN.PK 113–7D (Euroscarf, Frankfurt, Germany). Strains were grown in YPD medium (2% (w/v) D-glucose; 2% (w/v) peptone; 1% (w/v) yeast extract). If necessary, media was solidified by addition of 1.8% (w/v) agar.

Gas-chromatographic procedures

Determination of extent of conversion. After addition of *n*-butanol as an internal standard, extent of conversion was measured by means of GC/FID (HP 6890 GC equipped with an automatic liquid sampler). Separation was achieved on a CS-Carbowax CW 20M (CS-Chromtographie, Langerwehe) capillary column (50 m × 0.32 mm × 0.5 μm). The pressure of the carrier gas H_2 was 0.8 bar; the temperatures of injector and detector were 250 °C and 260 °C, respectively. The temperature program: 80 °C (0 min); 80 °C to 160 °C with 20 K/min; 160 °C (4.5 min); 160 °C to 180 °C with 40 K/min gave the following retention time : **1**: 6.4 min; **2**: 8.0 min; **3**: 11.0 min; *n*-butanol: 3.3 min; **7**: 6.8 min.

Determination of enantiomeric and diastereomeric excess. Gas-chromatographic separation of all isomers of **2** and **3** on a CS-Cyclodex β/IP (Chromatographie Service Langerwehe) capillary column (50 m × 0.32 mm) was achieved after derivatisation with

methoxylamine and trifluoroacetic anhydride. Cell-free samples were extracted three times with *tert*-butyl methyl ether (TBME), dried with $MgSO_4$ and evaporated to dryness. Methoximes of 5-hydroxy-2-hexanone were obtained after addition of 200 µL of methoxylamine hydrochloride (300 mM in dry pyridine) at 80 °C for 30 min. Excess pyridine was evaporated in a stream of nitrogen. Purification of methoximes was achieved by adding 500 µL of H_2O, saturated with NaCl and subsequent extraction with TBME. The combined extracts were dried over $MgSO_4$ and concentrated in a stream of nitrogen before 70 µL of trifluoroacetic anhydride were added carefully. After standing for 30 min at room temperature excess of trifluoroacetic acid and its anhydride was evaporated. Prior to injection the residue was diluted with CH_2Cl_2. Analysis was carried out on a HP6890 GC/FID equipped with an HP 5971 autosampler. The pressure of carrier gas H_2 was 0.48 bar; the temperatures of injector and detector were 250 °C and 300 °C, respectively. The temperature program: 50 °C (45 min); 50 °C to 100 °C with 20 K/min; 100 °C (15 min); 100 °C to 170 °C (5 min) resulted in the following retention times: **(2*S*,5*S*)-3**: 49.0 min; **(2*R*,5*R*)-3**: 49.3 min; ***meso*-3**: 50.3 min; **(2*S*)-2**: 52.2 and 52.7 min†; **(2*R*)-2**: 51.5 and 52.4 min‡.

Strain dependence of 2,5-hexandione reducing activity

After incubation of *S. cerevisiae* strains CBS 8066, CEN.PK 113–7D and L13 for 7 d in YPD cells were harvested through centrifugation (5 min, 5000g), washed with ice-cold 0.9% NaCl. Activity of 2,5-hexanedione-dehydrogenases was determined after disruption of the cells.

In order to determine enantioselectivity of the strains under study, 1% v/v 2,5-hexanedione was subjected to biotransformation employing the respective yeast strains. Conditions were equal to experiments with industrially produced compressed yeast as stated below. After complete conversion of **1** to **3**, the enantiopurity of the product was analysed by chiral-capillary GC/FID as stated above.

Stress induction

In order to expose exponentially growing cells to stress an overnight culture (OD_{600} = 5) was diluted to OD_{600} = 0.4 with fresh YPD and incubated for 2.5 h at 30 °C so that the culture was in exponential phase and an OD_{600} of 0.8 was reached. Cells were exposed to osmotic shock by addition of an equal volume of YPD containing 2 M sorbitiol (prewarmed to 30 °C). In order to expose the culture to heat shock, an equal amount of fresh YPD having a temperature of 44 °C (mixing temperature = 37 °C) was added. Stress induction through 2,5-hexanedione was studied after addition of 1% v/v (85 mM) of 2,5-hexanedione to an exponentially growing culture (OD_{600}=0.8). Cells were exposed to different stresses for 1 h at 30 °C or 37 °C (heat shock cells) with shaking (150 rpm). Subsequently cells were collected by centrifugation (5 min, 5000g) at 4 °C and washed once with cold 0.9% NaCl before being subjected to disruption and determination of enzyme activity. For experiments with resting non-growing cells, commercially supplied yeast cells (L13) were washed and suspended in PBS buffer pH 6.5 with 2% w/v glucose at an OD_{600} of 1.8. After preincubation for 1 h at 30 °C/150 rpm cells were subjected to osmotic stress by adding an equal amount of a prewarmed (30 °C) solution of 2 M sorbitol in PBS buffer pH 6.5 with 2% glucose to the culture. After an incubation period of 1 h and 10 h, respectively, cells were harvested and disrupted as described above in order to determine 2,5-hexanedione-reducing activity.

Enzyme assays

Disruption of yeast cells. Yeast cells were washed with cold 0.9% NaCl and resuspended in 0.1 M sodium phosphate buffer pH 7.0. After disruption according to ref. 70, enzyme activities were determined in cell crude extract. Protein was determined by the method of Bradford.[71]

Activity of dehydrogenases. Activity of dehydrogenases was measured spectrophotometrically by monitoring the decrease in absorbance at 340 nm with a ATI UNICAM UV4 spectrophotometer at 30 °C. The standard assay was carried out in phosphate-buffer 0.1 M pH 7.0 containing 0.2 mM NADPH and 0.2 mM $NADP^+$ for reduction and oxidation experiments, respectively. Concentration of substrate was 10 mM. The reaction was started by addition of the substrate. Apparent K_M and v_{max} were determined in a similar manner, whereas substrate concentrations were varied in a concentration range between 0.05 and 80 mM. The apparent Michaelis kinetic parameters were obtained by directly fitting the data to the rate equation of an enzymatic monosubstrate reaction by means of non-linear regression. One unit corresponds to the formation or consumption of 1 µmol NADPH per minute at pH 7.0 at 30 °C.

Glucose-6-phosphate activity. Glucose-6-phosphate assay was based on ref. 72. Enzymatic activity was measured in the presence of 20 mM $MgCl_2$ and 0.7 mM $NADP^+$ in 60 mM TRIS pH 7.6 using 2 mM glucose-6-phosphate as a substrate. The increase in absorbance at 340 nm was monitored spectrophotometrically with a ATI UNICAM UV4 spectrophotometer after the start of the reaction with 20–70 µL of cell extract. One unit catalyses the oxidation of 1 µmol glucose-6-phosphate per min at 30 °C in the presence of $NADP^+$ at pH 7.6.

Glyceraldehyde-3-phosphate activity. Glyceraldehyde-3-phosphate activity was measured in the reverse direction in a coupled system with 3-phosphoglyceric phosphokinase. Activity was assayed in 100 mM TRIS pH 7.6 with 6.7 mM 3-phosphoglycerate, 3.3 mM cysteine hydrochloride, 1.7 mM $MgSO_4$, 0.16 mM NADH, 1.1 mM ATP and 3.3 U/mL 3-phosphoglycerat phosphokinase. Decrease in absorbance at 340 nm was monitored with an ATI UNICAM UV4 spectrophotometer after the addition of 20–40 µL of cell extract. One unit catalyses the reduction of 1 µmol 3-phosphoglycerate per min in a coupled system with 3-phosphoglyceric phosphokinase at pH 7.6 at 30 °C.

Biotransformation of 2,5-hexanedione

Whole cell bioreduction. Whole-cell biotransformation of 2,5-hexanedione was carried out by suspending 125 g/L (wet-weight) of industrially produced compressed yeast in tap water containing 10% w/v sucrose. pH was kept constant at pH 6.3 by addition of

† Methoximation of 5-hydroxy-2-hexanone results in two diastereomeric methoximes per enantiomer; hence every enantiomer gives rise to two peaks.

1.5% w/v $CaCO_3$. Adequate aeration was achieved by adhering to a maximal air:culture ratio of 5:1. After 30 min of incubation (30 °C/150 rpm) 2,5-hexanedione was added, resulting in the stated concentration (standard concentration 1% v/v). Progress of the biotransformation was monitored by GC. To obtain (5*S*)-hydroxy-2-hexanone, biotransformations were stopped after 10 h, whereas (2*S*,5*S*)-hexanediol was obtained after 48 h of reaction. Cells were removed from the reaction mixture by centrifugation (5 min 5000g) and extracted three times with acetone. The cell free supernatant was concentrated *in vacuo* and subsequently treated with an excess of acetone in order to precipitate remaining cell fragments. After filtration acetone extracts were combined, concentrated *in vacuo* and dried over $MgSO_4$. After evaporation of the remaining acetone 5.2 g (66%) of raw product (containing 87% **2**) was obtained, as a clear, slightly yellow oil. Further purification of (5*S*)-hydroxy-2-hexanone was achieved through column chromatography on silica gel 60 (particle size: 0.045–0.06 mm; filling level: 27 cm; inner diameter of column: 2 cm) with ethyl acetate/n-hexane 1:1 v/v as eluent, resulting in 49% isolated yield.

Cell-free bioreduction with cofactor regeneration. Reduction of **1** with ADH-T employing substrate coupled cofactor regeneration was carried out in 0.1 M phosphate buffer pH 7.0 containing 80 mM 2,5-hexanedione, 2 M 2-propanol, 1 mM $NADP^+$ and 2 U/mL ADH-T. Reduction of **1** with a crude extract from *S. cerevisiae* L13 employing enzyme coupled cofactor regeneration through glucose/glucose dehydrogenase, was carried out in 0.2 M citrate-phosphate buffer pH 7.5 containing 120 mM D-glucose, 1 mM $NADP^+$, 50 mM 2,5-hexanedione, 0.8 U/mL 2,5-hexanedione reductase (added as crude extract) and 4.5 U/mL glucose dehydrogenase. The progress of the bioreduction was monitored by GC. Samples were obtained by withdrawing 100 µL from the reaction mixture and mixing it with 300 µL of ice-cold acetone containing *n*-butanol as an internal standard. After standing on ice for 2 h, samples were centrifuged (30 min, 10000g) and the supernatant analysed by GC/FID.

Extent of ad- and absorption on cells of *Saccharomyces cerevisiae* L13. 6.25 g wet-weight of industrially produced compressed *S. cerevisiae* L13, 5 g sucrose, 0.75 g $CaCO_3$ and 500 µL 2,5-hexanedione (4.36 mmol) were weighed in a 50 mL volumetric flask and filled with tap water to the graduation mark. A solution prepared in the same manner but without yeast, $CaCO_3$ and sucrose served as a control. Concentration of **1**, **2** and **3** was determined in the cell-free-supernatant after 48 h of biotransformation and compared to the control.

Simulations

Simulation was carried out using software COPASI.[69] Reversible Michaelis–Menten kinetics was assumed for both reactions. Kinetic parameters were taken from Table 2. The maximal velocities of both reactions were altered (v_{max}(**1**→**2**) = 1–50 fold v_{max}(**2**→**1**); v_{max}(**2**→**1**) = const.; v_{max}(**2**→**3**) = 1–10 fold v_{max}(**3**→**2**); v_{max}(**3**→**2**) = const.) and the resulting maximal concentration of the intermediate plotted against the ratio of v_{max}(**1**→**2**)/v_{max}(**2**→**3**).

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