

Enantioselective Microbial Reduction with Baker's Yeast on an Industrial Scale

Martin Bertau* and Martin Bürli

Abstract: Microbial synthesis is an important contribution to Green Chemistry and production-integrated environmental protection. The example of baker's yeast is used to demonstrate how microorganisms can be versatile reagents for asymmetric synthesis and how microbial technologies can be alternatives and complements to catalytic processes. The commercial viability of enantioselective microbial processes on an industrial scale is shown with the examples of (S)-3-hydroxybutyric acid ethylester (**1**), (2S,5S)-hexanediol (**2**) and (1R,2S)-cis-2-hydroxycyclohexane carboxylic acid ethylester (**3**). The investigation of several competing enzymatic pathways in the living cells during the reduction reaction allows the process to be controlled and makes this technology applicable for the large-scale commercial synthesis of **3**.

Keywords: Baker's yeast · Chiral pharmaceutical intermediates · Commercial chiral biosynthesis · Green chemistry · Microbial reduction

Microbes in Industrial Chemistry

Manifold applications for microbes in industrial chemistry exist. They serve as suppliers of products of value, active ingredients and enzymes. Of steadily growing importance are two additional applications: microbes as synthetic tools and microbes in exhaust air treatment. Bacteria, fungi or plant cells are utilised in biotechnology applications. Most processes work under sterile conditions; indispensable in the case of genetically engineered microorganisms. The search for applicable solutions for microbial conversions on a commercial scale led us to the use of baker's yeast (*Saccharomyces cerevisiae*) which is readily available, cheap, and, what has to be stressed, can be used under non-sterile conditions.

Baker's yeast is common in a series of industrial applications, the most familiar ones being brewing and wine-making or bakery. Yeast is also involved in bioethanol production [1].

At Rohner, baker's yeast serves as a reagent for organic-chemical syntheses, namely the production of chiral pharmaceutical intermediates for the life-science industry [2]. Thus, yeast technology allows elegant and easy access to a number of important key intermediates for enantioselective synthesis.

Bioreduction and Catalytic Hydrogenation

The synthetic potential of microbiological methods is commonly judged in comparison with asymmetric catalytic hydrogenation technology. Clearly, the limitation of biotechnological methods to often only one stereoisomer and the lower productivity is disadvantageous. Many conventional syntheses fail due to incompatibilities with the catalyst material. The harsh conditions often applied in conventional asymmetric synthesis are circumvented smoothly, allowing even sensitive compounds to be submitted to enantioselective transformations.

Stereoselective biohydrogenation of prochiral keto compounds is not the only type of reduction reaction for which ba-

ker's yeast is well suited. Moreover it is a multifunctional reagent, applicable for further stereoselective C=C reductions [3] and several C–C bond forming reactions [4]. The ensemble of catalytic hydrogenation and microbial methods covers a broader technological range than the single technologies themselves.

From this it is evident that microbial and catalytic asymmetric hydrogenation technologies complement each other in an ideal manner.

Yeast Technology: Green Chemistry

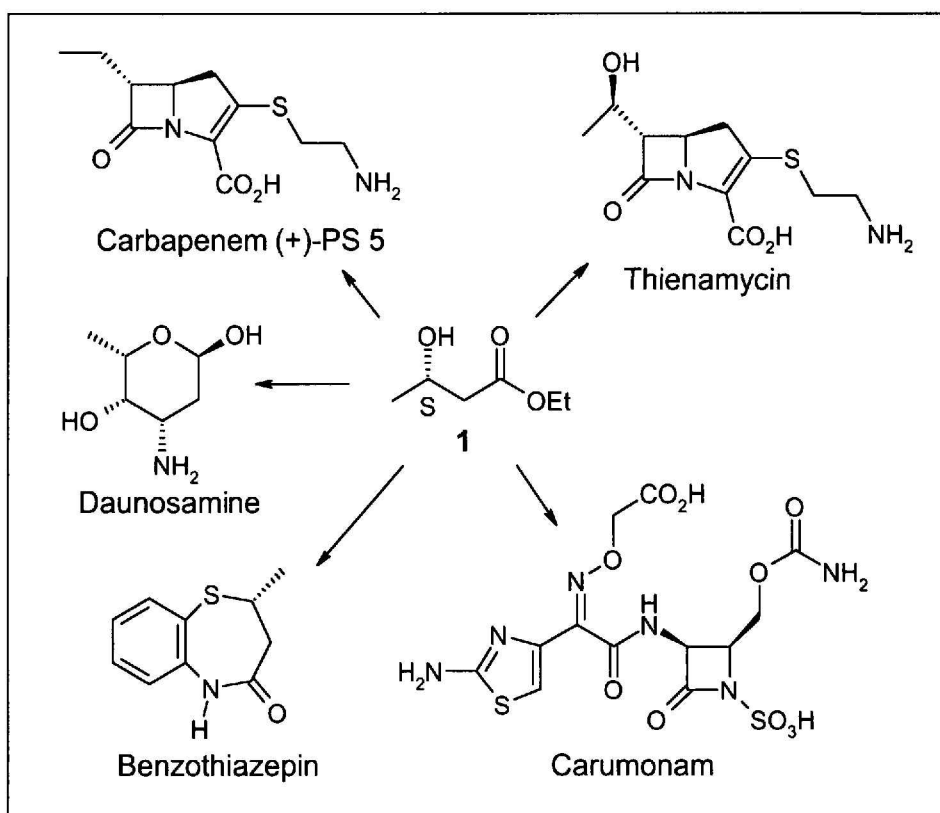
The use of baker's yeast in industrial chemistry is a form of production-integrated environmental protection [5] and it is a textbook example of how Green Chemistry is viable on a commercial scale. Only tap water, commercial baker's yeast and a carbon-source such as cane sugar or glucose are needed. Thus the fermentation process involves solely the regeneration of raw material. Efficient recovery systems allow a considerably lower loss of volatile organic compounds (VOC), rendering the downstream processing ecologically and economically attractive [6]. The ecological advantages of microbial syntheses with baker's yeast can be summarised as follows:

*Correspondence: Dr. M. Bertau
Rohner Ltd.
Chemical Development
Gempenstrasse 6
CH-4133 Pratteln
Tel.: +41 61 825 13 37
Fax: +41 61 825 13 25
E-Mail: mbertau@rohnerag.ch
<http://www.rohnerag.ch>

- Yeast and sugars regenerate raw material
- Water is an environmentally friendly solvent
- No or little organic loading (VOC) of exhaust air
- No or little wastewater pollution load
- Disposal of solid waste (biomass) is unproblematic
- Organic solvents are recovered to a high degree
- Chemistry free from heavy metals

Therefore, yeast chemistry is Green Chemistry.

All this demonstrates that microbiobiochemical conversions are also attractive from an economical point of view. The negligible pollution of air and water, high recovery degrees of VOC and the employment of readily available and cheap raw materials render this highly ecological technology inherently economical. Furthermore, instead of thermal treatment, organic exhaust air loads are degraded biotechnologically with a biofilter (Biovent®) [7].



Scheme 1: Hydroxybutyric acid ethylester (1) – chiral pharmaceutical precursor

Examples of Commercial Chiral Biosyntheses

The effective and powerful potential of microbial syntheses will be demonstrated with three examples of chiral building blocks:

1. The large-scale synthesis of (*S*)-3-hydroxybutyric acid ethylester (**1**) shows how a microbial process can be more cost-effective than a catalytic variant.
2. The case of (*2S,5S*)-hexanediol (**2**) is an example of how a microbial route is technologically superior to other variants and simultaneously the most economical method.
3. Finally, (*1R,2S*)-*cis*-2-hydroxycyclohexane carboxylic acid ethylester (**3**) is an example of a reduction that is only viable on a commercial scale by means of biotechnology.

Example 1: Bioreduction as a Cost-effective Variant

Hydroxybutyric acid ethylester (**1**) is a highly versatile pharmaceutical intermediate for chiral drug synthesis (Scheme 1).

The production of **1** by means of biotechnology is economically more favourable than production *via* catalytic hydrogenation. Due to its better space-time yield, the productivity of the catalytic

Table 1: Comparison of the biotechnological production of **1** vs. the catalytic alternative

Baker's Yeast	Catalytic Hydrogenation [8]
Water (500 ml)	Methanol (40 ml)
Baker's Yeast (50 g)	Ru-BINAP (0.4 g)
Sugar (100 g)	H ₂ (20–100 bar)
Ambient temperature (20–50 h)	Ambient temperature (40 h)
Three-step work-up	Two-step work-up
60–75% yield	98% yield
(<i>S</i>)-Enantiomer, ee >98%	(<i>R</i>)- or (<i>S</i>)-Enantiomer, ee >99%
Space-time yield: ~1.2 g l ⁻¹ h ⁻¹	Space-time yield: ~12.2 g l ⁻¹ h ⁻¹

variant increases on a very large scale (*e.g.* >1mt), as the costs for infrastructure and the catalyst material are compensated. Table 1 compares both alternatives.

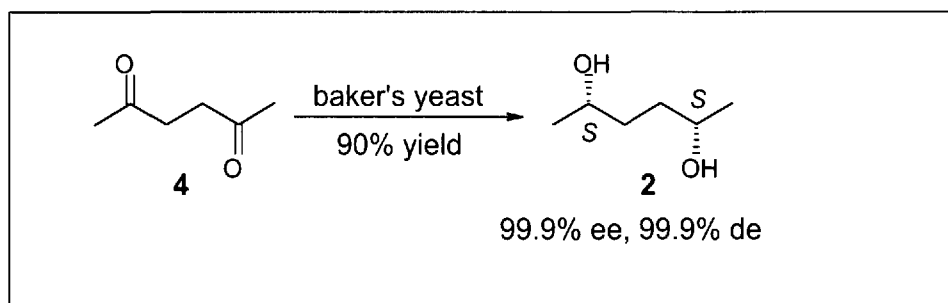
In the case of **1**, the limitation of the fermentation route to one stereoisomer is not disadvantageous, as the (*R*)-enantiomer *ent*-**1** is readily accessible by alcoholysis of (*R*)-polyhydroxybutanoate (PHB) [9]. The latter is also produced by microbial means – in this case as a bacterial energy store. Bacteria, such as *Alcaligenes eutrophus*, accumulate (*R*)-PHB to more than 90% of their dry cell-weight [10]. The synthesis of *ent*-**1** from (*R*)-PHB is less expensive than by fermentation pathways.

This is a clear example of how the limitation of only one stereoisomer can

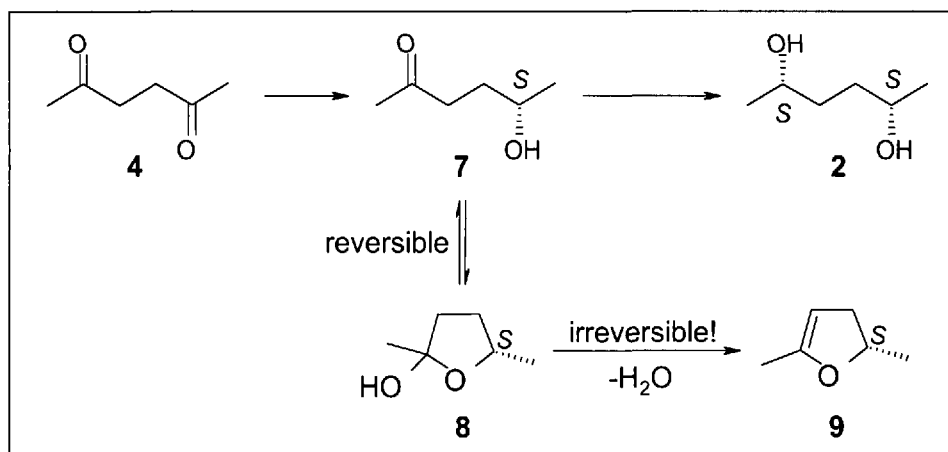
be overcome by the synergistic combination of biotechnological methods.

Example 2: More Competitive and More Efficacious than Alternative Processes

(*2S,5S*)-Hexanediol (**2**) serves as precursor of chiral five-membered ring heterocycles as ligands for asymmetric hydrogenation catalysts, such as DuPHOS [11][12]. For the production of **2** several procedures are described. The best reported catalytic route uses (*S*)-BINAP-Ru at 67 bar and affords a 4:1 mixture of the *cis*-product **2** and its *meso*-stereoisomer in only moderate yields directly from diketone **4** [13]. Other variants suffer from the high synthetic effort necessary and furnish **2** in unsatisfactory yields



Scheme 2: (2*S*,5*S*)-Hexanediol (**2**) – accessible in a stereopure form by microbial means on a commercial scale



Scheme 3: Side-product formation during reduction of **4**

and stereoselectivities [14]. All catalytic procedures have the common disadvantage that they are not commercially viable. Enzymatic methods involve racemic NaBH_4 -reduction of the diketone precursor **4**. The lipase-mediated stereoselective acylation of the (*R*)-configured alcohols furnishes the (*S,S*)-isomer in usually high ee, but in some cases in rather low diastereoselectivity [15]. Low conversion rates and long reaction times make enzymatic routes unfavourable.

A better alternative is a three-step procedure which circumvents the low stereoselectivity of the Ru-BINAP system for acetonylacetone (**4**) by using methyl acetoacetate (**5**) as a substrate. Saponification to 3-hydroxybutyric acid (**6**) is followed by a Kolbe-electrolytic decarboxylative dimerization to the respective hexanediol [16]. This process is suitable for the production of (*S,S*)-**2** and the (*R,R*)-enantiomer *ent*-**2** respectively. Although the enantiopurity of the product is high (ee >99%), this method suffers from the rather poor yield of the last step (55%).

The baker's yeast reduction of **4** furnishes **2** directly in a one-step process in up to 90% yield (Scheme 2). With an ee and de of 99.9% each, this process is cur-

rently the most powerful method to access enantiopure **2** in high yield and high purity (>99.5%). With only half the time effort and twice the yield of the catalytic alternative, the biotechnological variant produces **2** with almost 100% selectivity.

These results demonstrate how a biotechnological process can be ecologically attractive and economically superior to classical routes.

Selectivity

The formation of **2** is a highly selective process. Side-reactions are observed to only a marginal extent. Exact steering of the microbial process allows the circumvention of the competing ring-closure reaction of intermediate **7** to the five-membered ring species **8**. This side-path bears complications as the elimination of water to dimethyldihydrofuran **9** is irreversible (Scheme 3).

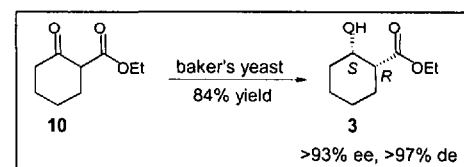
Example 3: Commercially Accessible by Biotechnological Means Only

(1*R*,2*S*)-*cis*-2-Hydroxycyclohexane carboxylic acid ethylester (**3**) has manifold applications, such as a precursor in natural product synthesis [17], for chy-

motrypsin inhibitors [18] and for carbenams [19]. There are currently three technologies for the synthesis of **3**. The asymmetric hydrogenation of the β -ketoester precursor **10** furnishes **3** with only poor stereoselection (14% ee) as a mixture with its (1*R*,2*R*)-diastereomer **12** [20]. The enzymatic variants are laborious. After reduction of **10** with sodium borohydride and acylation of the resulting four stereoisomers, treatment with lipases recovers **3** from the reaction mixture in 35–50% yield (45–50% conversion) with 86–95% ee [21]. The long reaction times before workup of 3–10 days and the low conversion rates are prohibitive for commercial applications.

Yeast technology furnishes **3** in a multi-kilogram scale after complete conversion in 70% yield and good stereoselectivity (>93% ee, >97% de, Scheme 4).

The advantages of the bioprocess result from the short reaction times and the high selectivity and yield. To our knowledge, bioreduction with baker's yeast currently is the best technology for producing **3**. Needless to say, this process is working well commercially.

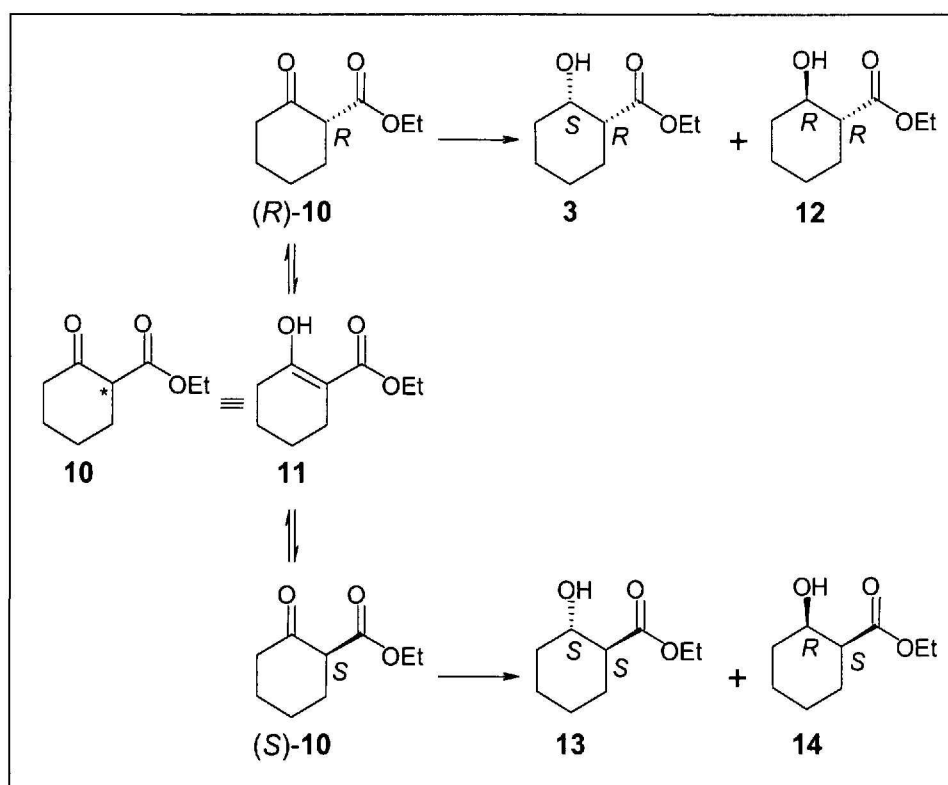
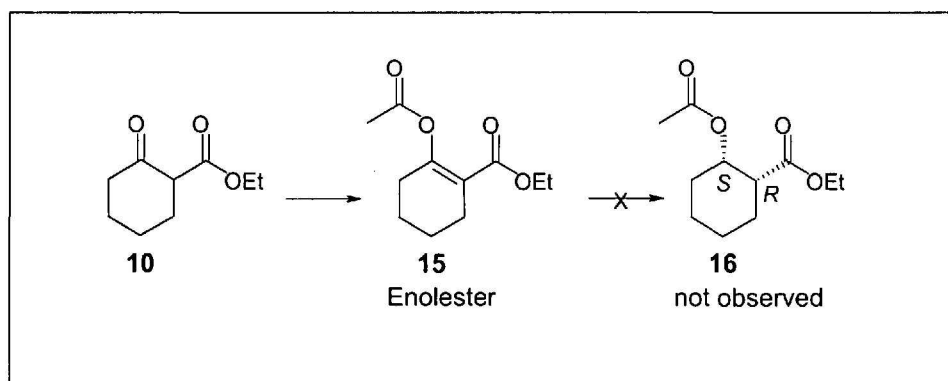


Scheme 4: Baker's yeast reduction of **10**

Bistereogenic Asymmetric Bioreduction

The impressive enantioselectivity of enzyme-mediated stereoselective reductions in living cells is finely represented by the two examples mentioned above which have in common that only one stereocentre is generated per reduction step. The bioreduction of acetonylacetone (**4**) to hexanediol (**2**) has to be regarded as a monostereogenic reduction, as the two keto groups are reduced subsequently. Moreover, they are not positioned vicinally, hence there is no influence of the keto–enol tautomerism on the outcome of the stereoselection of the reduction reaction.

The bistereogenic enantio- and diastereoselective formation of α -substituted β -hydroxyesters has attracted much attention because of their widespread applicability in biologically active substance synthesis [22]. Although a variety of chemical methods has been developed for the enantioselective preparation of α -

Scheme 5: Stereoselectivity of the reduction of **10**Scheme 6: No enol-reduction of the enolester **15**

substituted β -hydroxyesters, the situation is unsatisfactory. From many procedures like α -alkylation of β -hydroxyesters, the *anti* alkylated esters are usually obtained. The optically active *cis* esters are still difficult to prepare [23].

Whereas the introduction of one stereogenic centre is rather facile, the outcome of the baker's yeast reduction of α -substituted β -oxocarboxylic acid derivatives is more complex, since in this case the substrate becomes chiral and both enantiomer and diastereotope selectivities are possible (Scheme 5).

In order to elucidate the mechanism of this reaction, it was investigated whether the reduction represents a carbonyl reduction of a chiral β -ketoester, or

whether there is a *cis*-reduction of the enol **11**. For this purpose, the enol was trapped as an acetate **15** and the latter was submitted to microbial reduction. However, no reaction was observed, which is in line with the findings of Ohta and co-workers [24]. Hence it follows that the reaction proceeds *via* carbonyl reduction (Scheme 6).

In the present case, reduction takes place predominantly on the (*R*)-configured β -ketoester (*R*)-**10**. The stereoselectivity of this reaction is impressively high, as **12** is normally not observed. However, if the described reaction pathway were the only one followed, **3** would be enantiomerically pure, since no enantiomer **14** can be formed along this route.

However, both **13** and **14** are observed in the crude product mixture. Obviously, competing pathways or less stereoselective enzymes exist that also reduce the (*S*)-configured ketoester (*S*)-**10**. Remember (*R*)-**10**, **11** and (*S*)-**10** are members of a fast-establishing equilibrium.

The answer is given by the observation that **3** is reoxidized by living yeast cells, whereas **3** is stable under oxidizing conditions in the absence of microbes. Moreover, if pure **3** is added to baker's yeast, not only the reoxidized ketone **10** is observed, but also significant amounts of **13** and **14** are formed. This strongly indicates that the product **3** itself induces a competing enzymatic pathway giving rise to the reduction of (*S*)-**10** to the stereoisomers **13** and **14**.

For these reasons, possibilities for the selective inhibition of the reduction of (*S*)-**10** were investigated. An inhibition of the reoxidation reaction makes no sense, as the presence of unreduced ketone **10** would result in the formation of **13** and **14** in any case, once this pathway is open.

A comparison of the reduction of **10** with and without the presence of an inhibitor is given in Table 2.

Table 2: Influence of inhibitor on the selectivity of the bioreduction of **12**

%	3	12 ^{a)}	13	14
inhibited	93.8	-	3.4	2.8
non inhibited	80.1	-	17.0	2.9

a) **12** is not observed (see text)

As shown in Table 2, there is no change in the relative amounts of **14**, whereas the formation of **13** from (*S*)-**10** could be reduced markedly by *ca.* 75%. From these results it is evident that at least one more enzyme must be involved in (*S*)-**10** reduction, as otherwise the formation of **14** would have been influenced in a comparable way as **13**. Therefore, the inhibition is effective exclusively for the conversion of (*S*)-**10** into **13**. The competing enzymatic reduction of (*S*)-**10** to **14**, however, remains totally unaffected.

Selectivities

Besides the rather complex matter of stereoinduction in the reduction of **10**, there are side reactions which also have an influence on the total yield of **3**.

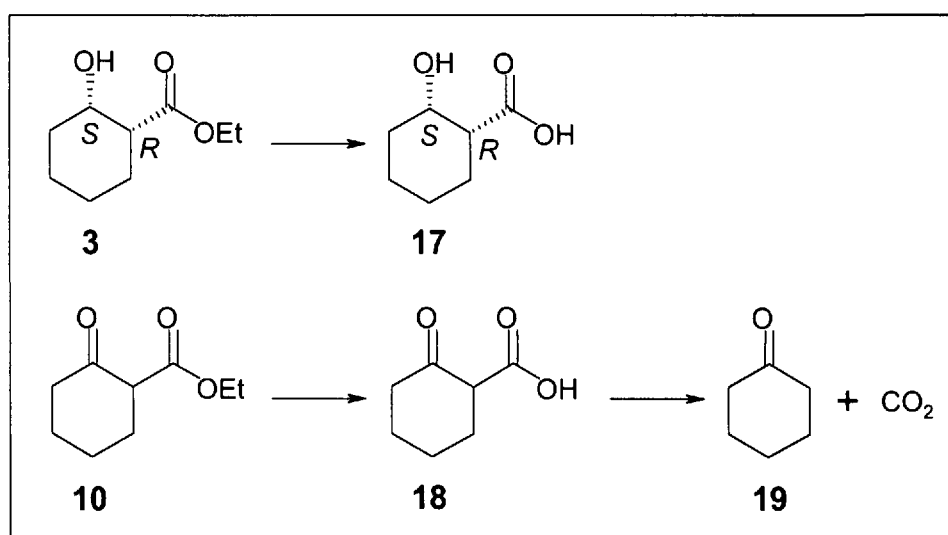
The most important side reaction is ester hydrolysis. The saponification of **3** and **10** leads to the respective acids **17** and **18** of which the β -ketoacid **18** decarboxylates spontaneously to give cyclo-

hexanone **19** (Scheme 7). The choice of the proper reaction conditions allows these by-products to be suppressed to an insignificant extent.

Conclusions

The three examples have shown that biotechnological processes are indeed viable in commercial industrial chemistry. Example 3 has shown that microbial reactions can be understood to a great extent and thus easily steered and controlled. With its consumption of regenerative raw material only and a mostly complete regeneration of volatile organic compounds, such as solvents for extractions, microbial synthesis is an important contribution to Green Chemistry and production-integrated environmental protection. Yeast is a versatile reagent for asymmetric synthesis and therefore microbial technologies can be both alternatives and complements to catalytic processes. With regard to all this, biotechnological reactions are of steadily growing acceptance and their meaning in industrial Green Chemistry will increase.

Received: June 13, 2000



Scheme 7: By-product formation during enantioselective microbial reduction of **10**

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