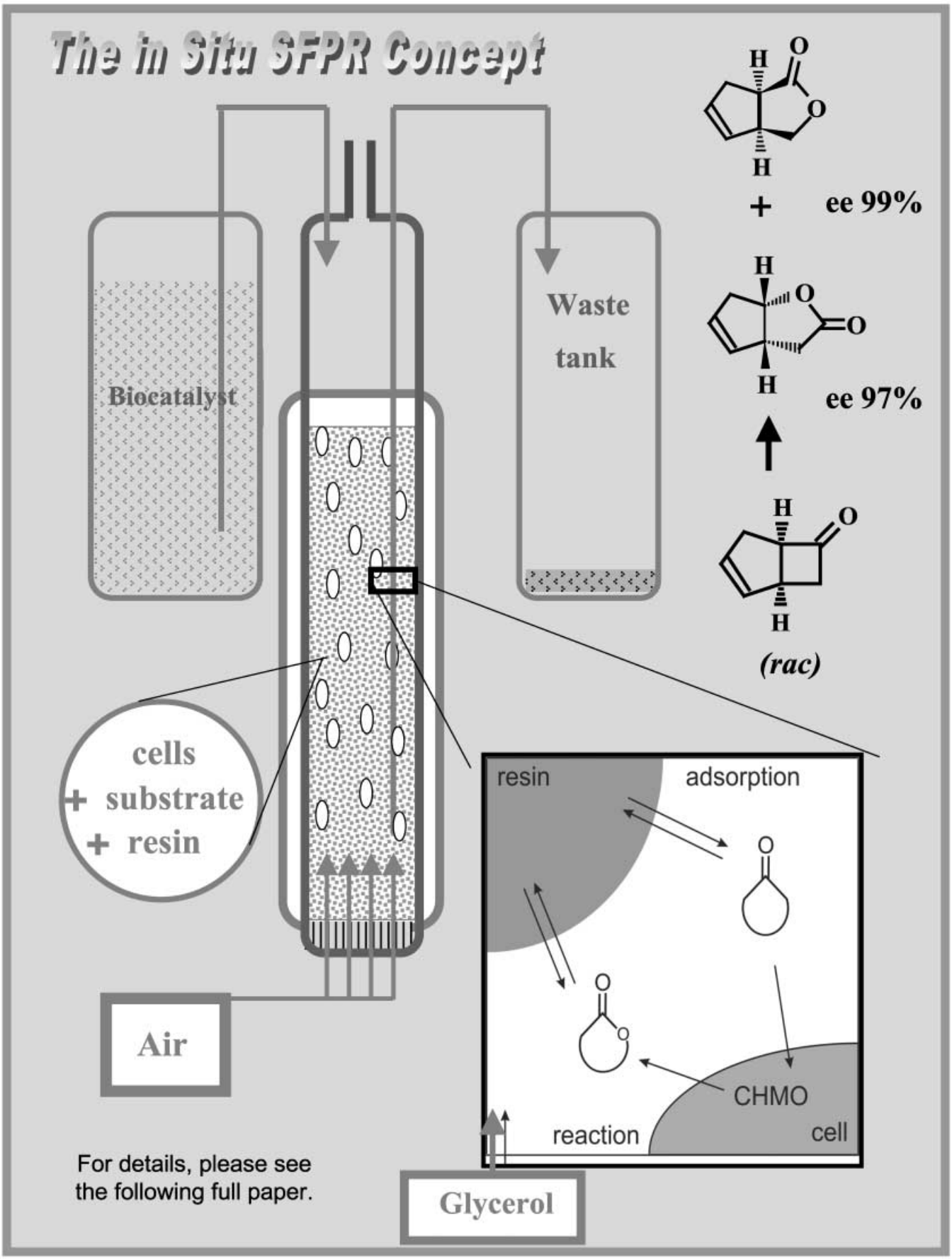

 DOI: 10.1002/adsc.200303183

Microbial Transformations, 56. Preparative Scale Asymmetric Baeyer–Villiger Oxidation using a Highly Productive "Two-in-One" Resin-Based *in situ* SFPR Concept

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Received: October 20, 2003; Accepted: January 17, 2004

Abstract: An efficient preparative scale process for achieving asymmetric Baeyer–Villiger oxidation – a reaction still very difficult to perform using conventional chemistry – is described. This process is based on a biocatalytic whole cells strategy – using a recombinant *E. coli* strain overexpressing cyclohexanone monooxygenase (CHMO) – combined with a "two-in-one" *in situ* "substrate feeding and product removal" concept (SFPR) using an adsorbent resin. The most efficient resin out of fourteen tested was used in three types of bioreactors (conventional, recycle and bubble column) that were compared. The best one proved to be the bubble column reactor, where 25 g (0.23 M) of *rac*-bicyclo[3.2.0]hept-2-en-6-one could be totally transformed using a one-litre vessel with a volumetric productivity of about 1 g L^{-1} h^{-1} (i.e., 7.7 mmol L^{-1} h^{-1}). This led to the production of the two corresponding regioisomeric lactones, which were both obtained in excellent enantiomeric purity (ee > 98%) and high preparative yield (84%). To our knowledge, these results represent the best example of a (highly productive) preparative scale asymmetric Baeyer–Villiger oxidation.

Keywords: adsorbent resin; asymmetric Baeyer–Villiger oxidation; extractive biocatalysis; *in situ* substrate feeding and product removal concept; whole cell biotransformation

Introduction

Asymmetric synthesis processes have been considered for years as essential challenges for fine organic synthesis, as exemplified by the attribution of the 2001 Nobel Price of Chemistry to three pioneers in this field.[1] However, in spite of world wide efforts, several types of "fundamental" organic reactions still are very difficult, if not impossible, to be performed using conventional chemistry with satisfactory results, i.e., allowing: a) large substrate acceptance, b) high enantioselectivity (if starting from a racemic substrate) or enantiotoposelectivity (if starting from a prochiral substrate), and c) experimental conditions providing reasonable (potentially industrial) scale-up opportunities. Such a reaction is the Baeyer–Villiger (BV) oxidation, whose asymmetric version has only been described with limited success as far as the three above-cited criteria are concerned.[2] Interestingly, much more satisfactory results have been described for over two decades using a biocatalytic approach. Research in this field covered the identification of racemic and prochiral substrates,[3] the exploration to potential application for chemical synthesis[4] as well as the identification and characterisation of novel Baeyer–Villigerase genes[5] and the elaboration of analytical assays for their detection.[6,7] The state of the art on biocatalysed asymmetric BV oxidation, and particularly the analysis of the problems still to be solved for implementing this reaction to preparative/pilot plant scale, have been recently discussed.[8] This study illustrated clearly the need for further elaboration of an efficient process allowing extrapolation to "large scale" application of this reaction.

The best known and rather well characterised Baeyer–Villigerase is cyclohexanone monooxygenase (CHMO) from the bacteria *Acinetobacter calcoaceticus* NCIMB 9871. This flavoenzyme uses molecular oxygen to carry out oxidation and is NADPH-dependent, which makes cofactor recycling necessary. Although some approaches have been described using purified CHMO with an *in situ* NADPH recycling system (e.g., engineered formate dehydrogenase[9] or alcohol dehydrogenase[10]), it clearly appeared over the years that the use of whole cells – which ensure cofactor recycling by the cell metabolism itself – is still the simplest and most

  DOI: 10.1002/adsc.200303183

promising way for large scale applications. However, employing the wild-type strain brings about disadvantages like low CHMO activities, low optimum substrate concentration and the handling of a class 2 pathogen microorganism.[11] Moreover, a lactone hydrolase is present in this strain, leading for some substrates to product degradation and therefore an important drop of yield.[12] In order to overcome these problems, cloning and overexpression of CHMO has been achieved in different hosts.[5a] One of them is *E. coli* TOP10(pQR239).[13] This non-pathogenic strain is comparatively fast growing and does not contain lactone hydrolase. A high CHMO content is reached after induction by the rather cheap L-(+)-arabinose.

Up to now, only scarce descriptions of preparative scale microbial Baeyer–Villiger processes can been found in literature.[14,15] They used a recombinant strain of *E. coli* in a whole cell biotransformation and operated a fed batch fermenter – either by continuous or portionwise addition of the respective substrate. Both also employed a product removal technique based on adsorption of the products on a solid support after completion of the biotransformation. However, these attempts led to rather low productivity, due to loss of activity with increasing product concentration in the cell broth, as well as to deactivation and probably cell death after 8 – 10 hours.

The aim of our work was to provide a more universal, productive and efficient process for microbial BV oxidation, thus to open the way to scale-up extrapolation. In particular, we wanted to address the typical problems of inhibition, cell toxicity or substrate solubility. The basic idea, already exemplified for very elegant – but scarce – biocatalysed reduction reactions,[16] was to combine at the same time **both** *in situ* substrate feeding **and** *in situ* product removal in a whole cell biotransformation, using an adsorbent resin. This particular type of "extractive biocatalysis"[17] could be called a "two-in-one" resin-based *in situ* substrate feeding and product removal (*in situ* SFPR) process. In preliminary results,[18] we had successfully explored the possibility to set up such a gram-scale experiment by using an appropriate adsorbent material. We describe here the optimisation of this approach, its implementation at larger scale and the comparison of three different types of bioreactors.[19]

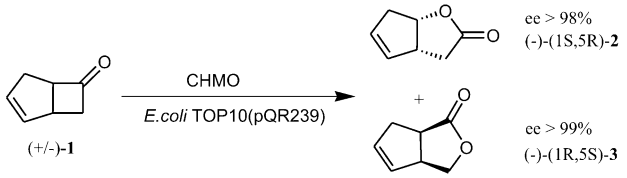
Results and Discussion

Properties of the Test Substrate

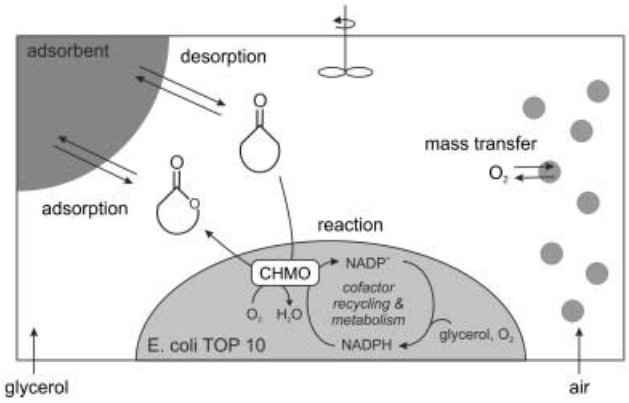
Microbial Baeyer–Villiger oxidation of commercially available *rac*-bicyclo[3.2.0]hept-2-en-6-one [(**1**) a substrate which became, over the years, the favourite test substrate for this type of reaction] was chosen as a model reaction to explore the applicability of the resin-based *in situ* SFPR concept. As we had described previously, this racemic ketone was surprisingly oxidised into two different regioisomeric lactones, both being nearly enantiomerically pure (Scheme 1).[20] In fact, the two enantiomers of **1** reacted with a specific but different regioselectivity: the (+)-ketone yielded the (expected) lactone **2** while the (−)-ketone was transformed to the (unexpected) lactone **3**. Bicyclo[3.2.0]hept-2-en-6-one (**1**) is fairly soluble in water (about 15 g L^{-1}) and, due to their higher polarity, the solubilities of **2** and **3** are somehow better. Previous studies, aimed to explore BV oxidation of **1** with whole cells of recombinant *E. coli* TOP10(pQR239), showed an inhibitory effect of **1** above 0.4 g L^{-1}.[21] Half of specific whole cell activity was already lost at a ketone concentration of 1 g L^{-1}. As well, **2** and **3** inhibited the enzymatic reaction if their total concentration exceeded 2 – 3 g L^{-1}. Moreover, it has been described previously, using the purified enzyme, that an increase of substrate concentration lowers the ee of the obtained "expected" lactone **2**.[22] Thus, reactant and product concentrations in the liquid phase have to be controlled accurately to maximise cell productivity and enantioselectivity. This was explored by using an adsorbent material loaded with ketone **1** prior to biotransformation.

An illustration of the entire "two-in-one" process going on in the cell broth is given in Scheme 2. Thus, when adsorbent (pre-loaded with **1**) is added to the cell broth, some substrate desorbs into the liquid phase and can be further transported into the cell, where it will be oxidised by the overexpressed CHMO. The produced lactone(s) then leaves the cell, goes into aqueous solution and is further re-adsorbed onto the solid support. Owing to the above-mentioned inhibition problems, it is of utmost importance that the load of the adsorbent material is adjusted to values leading to a concentration (in the aqueous phase) below the inhibiting level for both substrate and product. This can be tuned according to the adsorption isotherms of substrate and product for a specific support (see below). In the course of the enzymatic reaction, cofactor recycling and other parts of the cell metabolism have to be ensured by aeration of the system and glycerol feeding; their shortage leading to an early activity loss.

To be appropriate for the "two-in-one" *in situ* SFPR concept, a resin needs to provide a high capacity or a



Scheme 1. Microbial Baeyer–Villiger oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one (**1**).



Scheme 2. Principle of the "two-in-one" resin-based *in situ* SFPR process for microbial Baeyer–Villiger oxidation.

high load at the equilibrium concentration chosen for the biotransformation. The load X is defined as:

$$X = \frac{m_i}{m_{\text{dry ads.}}} \quad (1)$$

where m_i is the weight of the compound i and $m_{\text{dry ads.}}$ is the dry weight of the adsorbent. A high load reduces the mass of adsorbent needed per gram of substrate. Moreover, adsorption and desorption need to proceed rapidly to avoid substrate limitation or product accumulation in the liquid phase. It is expected that ketone and lactone show rather the same adsorption behaviour, albeit the lactone should be less adsorbed to the adsorbent than the ketone because of its slightly higher polarity. According to the inhibition studies discussed above, the initial ketone working concentration in the aqueous phase was set to 0.5 g L^{-1}.

Screening of Adsorbent Materials

In order to distinguish the resin properties most influencing the adsorption behaviour of bicyclo[3.2.0]-hept-2-en-6-one (**1**), we screened different commercially available adsorbents, combining different materials, different mean pore diameters and different inner surfaces (Table 1). Our results showed that the adsorption equilibrium measured for these different adsorbents could arbitrarily be divided into two groups: those with a Γ-shaped curves (Figure 1) and those with an S-shaped curves (Figure 2). We defined a working load, X^{eq}, which corresponds to the optimal initial ketone concentration for a biotransformation of 0.5 g L^{-1}. When comparing the inner surface to the attainable working load, a direct relation of adsorption performance and inner surface of the adsorbent was observed. The influence of the other parameters appeared to be less pronounced.

Duolite S761, a rather polar material, was found to be the less efficient resin, but it also had the smallest inner volume and surface as well as the biggest pores. Amberlite XAD 2, with much smaller pores and consisting of a hydrophobic polyaromatic material, showed a better adsorption for a same inner surface, whereas Amberlite XAD 7 exhibited a slightly lower quality than XAD 2 at $c^{eq} = 0.5$ g L^{-1}, even if its inner surface was 1.5 times higher. This is probably due to the higher polarity of its material (acrylic ester). For the other resins exhibiting a Γ-shape in the adsorption diagram, adsorption efficiency increased slightly with growing inner surface.

A significant improvement was achieved by the materials having a pronounced S-shape adsorption curve. The S-shape indicates a much better performance in the low equilibrium concentration region ($c^{eq} < 5$ g L^{-1}).

Table 1. Adsorbent materials and their properties.

Adsorbent	Material	Mean pore diameter [Å]	Inner surface [m^2/g]	Working load X^{eq} at $c^{eq} = 0.5$ g L^{-1}
Γ-shaped adsorption curves:				
Duolite S761	methylol	600	300	< 0.03
Amberlite XAD 2	polyaromatic	105	300	0.07
Amberlite XAD 7	acrylic ester	40	450	0.05
Sepabeads SP 207	brominated styrene	105	650	0.12
Amberlite XAD 1180	polyaromatic	400	> 500	0.11
Optipore L323	polyaromatic	100	650	0.1
Amberlite XAD 4	polyaromatic	40	725	0.13
Lewatit VPOC 1064 MD PH	styrene-DVB	50 – 300	700 – 800	0.15
Amberlite XAD 16	polyaromatic	100	800	0.15
S-shaped adsorption curves:				
Active charcoal Norit Darco	charcoal	–	–	0.17
Active charcoal	charcoal	–	–	0.25
Active charcoal Norit RO 0.8	charcoal	–	–	0.3
Optipore L493	polyaromatic	46	1100	0.3
Lewatit VPOC 1163	styrene-DVB	5 – 100	1000 – 1400	0.31

X^{eq} = equilibrium load of adsorbent, c^{eq} = corresponding equilibrium concentration.

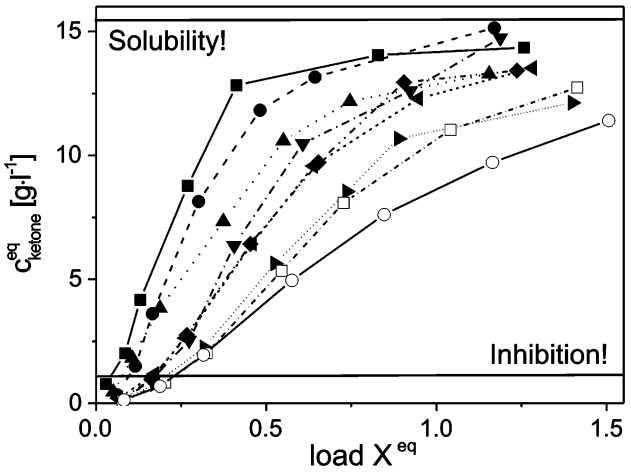


Figure 1. Adsorption equilibria of bicyclo[3.2.0]hept-2-ene-6-one (**1**) on different materials (Γ-shaped curves): Liquid phase concentration c^{eq}_{ketone} in dependence of the equilibrium load X^{eq}. (■) Duolite S761, (●) Amberlite XAD2, (▲) Amberlite XAD7, (▼) Sepabeads SP207, (◆) Amberlite XAD1180, (◀) Optipore L-323, (▶) Amberlite XAD4, (□) Lewatit VPOC1064, (○) Amberlite XAD16.

The low-priced active charcoal gave comparatively high loads at the required working concentration. Unfortunately, its poor mechanical stability led to abrasion and thus makes it unattractive for biotransformation.

Apparently, the working load of the different adsorbent materials is not related to the mean pore diameter. This parameter, or more exactly, the pore size distribution of an adsorbent which determines this mean value, is expected to influence the velocity of adsorption or desorption since they proceed mainly *via* intra-particle diffusion.[23] However, kinetic measurements were not part of this study as adsorption and desorption rates were presently not limiting in the biotransformation. The best candidates finally turned out to be two macroporous resins, Optipore L-493 and Lewatit VPOC 1163, which exhibit the highest inner surface and small pore diameters. They showed a similar, pronounced S-shape in their adsorption curve and allowed a working load of 0.3 (i.e., allowing to adsorb 10 g of substrate on 30 g of resin) at $c^{eq} = 0.5$ g L^{-1}. In the following studies, only Optipore L-493 was used.

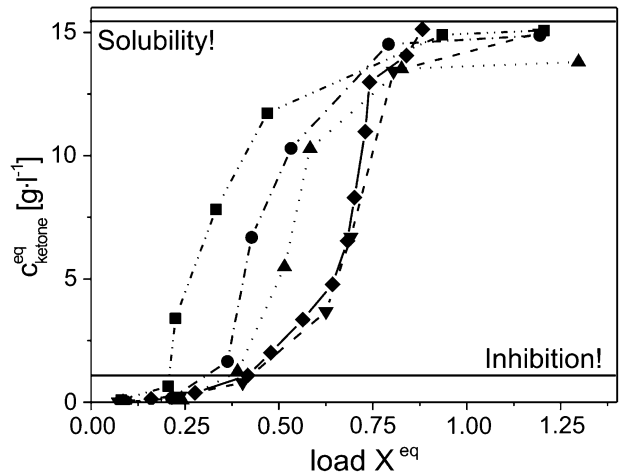


Figure 2. Adsorption equilibria of bicyclo[3.2.0]hept-2-en-6-one (**1**) on different materials (S-shaped curves). Liquid phase concentration c^{eq}_{ketone} in dependence of the equilibrium load X^{eq}. (■) active charcoal Norit Darco, (●) active charcoal, (▲) active charcoal Norit RO 0.8, (▼) Lewatit VPOC1163, (◆) Optipore L-493.

Preparation of the Biocatalyst

Prior to biotransformation, *E. coli* TOP10(pQR239) was grown in a 10-L fermenter under the appropriate conditions. The CHMO production takes place in the late log phase.[15] Typical yields are 3.5–6 g L^{-1} dry weight of cells and the average specific whole cell activity [measured against bicyclo[3.2.0]hept-2-en-6-one (**1**) as described in the Experimental Section] was about 80 U g^{-1}. The glycerol concentration in the medium at the end of the fermentation was usually about 4 g L^{-1}, and was readjusted to 10 g L^{-1} by addition of glycerol before biotransformation. To carry out the biotransformation at higher cell concentrations, part of the broth was centrifuged and the obtained cells were resuspended in the remaining broth.

Choice of the Reactor

Several possibilities can be imagined for setting up a "two-in-one" process at a laboratory scale. Thus, the biotransformation can be performed: a) in a conventional fermenter, with cells and adsorbent mixed by mechanical agitation, b) using a recycle reactor where the resin is placed into an external fixed bed column connected to a fermenter. The latter case might be especially helpful if an important amount of resin is needed to bind the substrate (which leads to an unfavourably high resin/liquid phase volume ratio). c) A third alternative could be to use a bubble column reactor containing the resin,[18] which would offer better aeration due to the higher gas-liquid interface. Mechanical stress also should be reduced in this type of reactor, cells and resin dispersion being assured by the rising of bubbles only.

We have tested and compared the course of the biooxidation of **1** using these three types of reactors and the results are described below. All preparative scale experiments were carried out at a 1-L scale using for each batch 25 g (0.23 mol) of *rac*-**1**. To ensure comparability, the air flow rate was kept constant at 3.3 vvm in all the reactor set-ups.

Use of a Conventional Reactor

The time course of an experiment conducted in a conventional (1.5-L) fermenter is shown in Figure 3. The concentrations of ketone **1** and of combined lactones **2** and **3** in the liquid phase, as well as the proportion of combined lactone adsorbed on the resin, are displayed. As expected from our Optipore L-493's adsorption equilibrium studies described above, the ketone concentration in the aqueous phase was initially about 0.5 g L^{-1} and decreased with time (i.e., conversion ratio), while the combined lactone concentration increased nearly linearly, without exceeding 1.2 g L^{-1}. This shows that the process was not limited by the desorption step under the given reaction conditions, because enough substrate was always available in the liquid phase. On the other hand, the risk of product inhibition was minimised since lactone concentration was largely below 2 g L^{-1} even at the end of the biotransformation. This means that the adsorption equilibrium of the lactones was as favourable as estimated and that adsorption was fast enough to avoid lactone accumulation in the cell broth.

The proportion of lactones on the resin increased linearly during the first 20 hours and about 55% of lactones were obtained on the resin. However, after 24 hours, the biocatalyst's activity was lost. Therefore, in order to bring oxidation to completion, the resin was filtered off, the cell broth was discarded and a fresh cell broth (conserved at 4 °C) was put into the fermenter. The moment of biocatalyst exchange is indicated in Figure 5. In the second cycle, the proportion of lactones on the resin raised to 83%. The loss of compounds (substrate and product) due to this exchange was low, most of them being bound to the resin and only a small amount being solubilised in the aqueous phase.

The DOT (dissolved oxygen tension) profile in the fermenter during the biotransformation is shown in Figure 9. It was constantly near zero when the cells were active. Oxygen concentration in the liquid phase went up only at the end of each cycle, indicating deactivation and probably cell death. Even though aeration at 700 min^{-1} of agitational speed and 3.3 vvm of air was maximised, the biotransformation was characterised by a strong oxygen limitation.

Use of a Recycle Reactor

A second type of reactor form could be a so-called "recycle reactor" as illustrated in Scheme 3. Here the charged resin was placed as a fixed bed into a column in an external loop, a set-up that should be useful in case of poor adsorption of a substrate onto the adsorbent material. Indeed, a low working load would imply a high quantity of resin and thus a high volume resin/liquid phase ratio, which means that a homogeneous dispersion of the resin in the fermenter would become difficult, if not impossible.

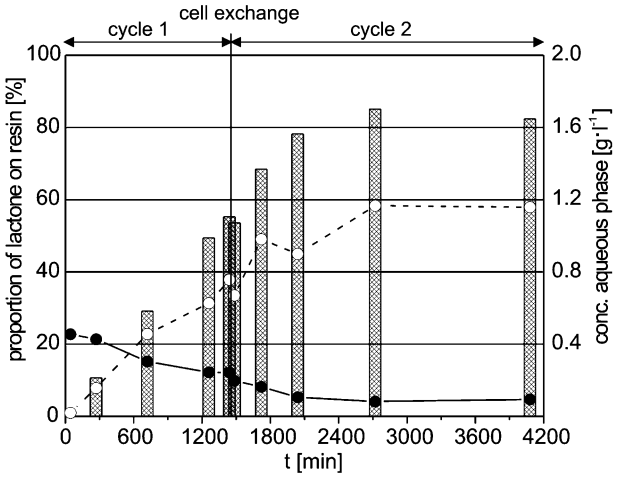
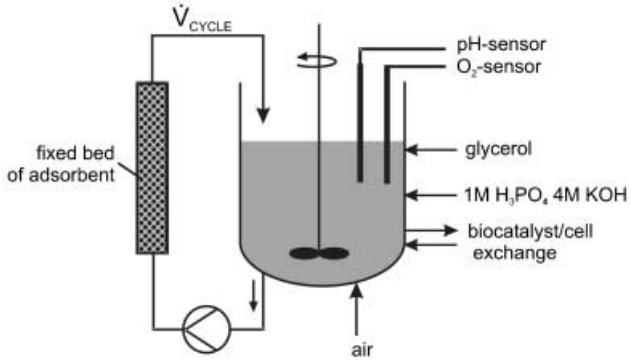


Figure 3. Baeyer–Villiger oxidation of 25 g of *rac*-**1** in a 1-L fermenter over two cycles (exchange of biocatalyst after 24 hours). ▨ proportion of readsorbed combined lactones extracted from a resin sample, ● ketone and ○ lactone concentration in the cell broth.

Cell broth was passed through the resin by a peristaltic pump. Circulation in the loop had to be fast enough to assure a sufficient substrate supply to the cells and a fast removal of the formed lactones from the fermenter part of the reactor. Moreover, cells in the loop being not aerated, a too long residence time could lead to a lack of oxygen and has to be avoided. Thus, the flow was set to give a residence time in the column of about 2 min which is equivalent to a total circulation time of the liquid phase of 10 min. During biotransformation, the chosen recycle flow rate was able to provide sufficient amounts of substrate to the cell broth in the fermenter. In Figure 4, the time course of the ketone concentrations at the entry of the fermenter (outlet of the column) and in the fermenter itself are shown. They both decreased as the biotransformation proceeded. The concentration at the outlet of the column was twice as high as the one



Scheme 3. Recycle reactor with fixed bed of adsorbent in external loop.

inside the fermenter, indicating ketone consumption. Since ketone **1** was always sufficiently present in the fermenter, desorption was never a rate-limiting step. The initial ketone working load in this experiment was 0.5 which should correspond to an equilibrium concentration in the liquid phase of 2 g L^{-1}. The exchange over the column being not fast enough to attain equilibrium, the liquid in the entry of the fermenter contained only about 1 g L^{-1} of **1**. Moreover, because ketone was always consumed, its aqueous concentration decreased in the fermenter to reach about 0.5 g L^{-1}, a value which allows us to avoid substrate inhibition.

The biotransformation in the recycle reactor proceeded fast and led to 85% of combined lactones (**2** and **3**) on the resin in less than 20 hours (Figure 5). This result could be, to some extent, explained by the increase of cell concentration to 10 g L^{-1} dry weight employed in this experiment. Therefore, a comparison with the other experiments conducted at 6.4 g L^{-1} dry weight is not directly possible. Nevertheless, this experiment showed clearly that BV oxidation was feasible in such a recycle reactor and that *E. coli* cells tolerated being pumped through the fixed bed. After 48 hours of recycling, however, some aggregation of cells around the resin particles was observed, forming a paste which could lead to severe clogging over time. As in the case of a conventional reactor, CHMO activity of the cells was lost after approximately 24 hours. This activity decrease (and probably cell death), was visible from the increase of the DOT of the cell broth (Figure 9) which was near zero as long as the reaction proceeded, and then increased linearly. The higher working load of the adsorbent resin led to a combined lactone concentration of 2.2 g L^{-1} in the aqueous phase at the end of biotransformation. Because this value was close to the product inhibition level (*ca.* 2.5 – 3 g L^{-1}), we did not carry out a new cycle by exchanging the biocatalyst.

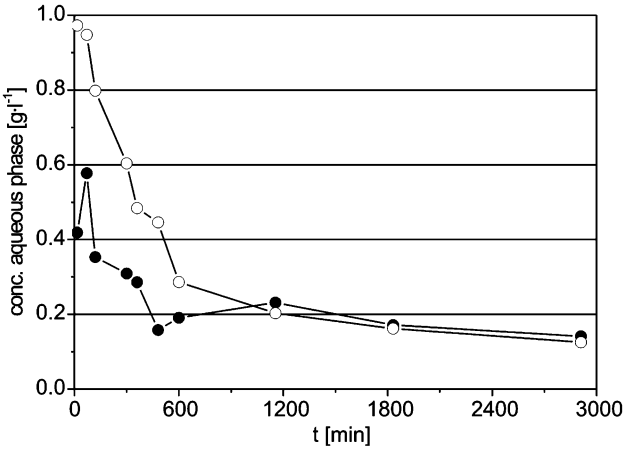


Figure 4. Baeyer–Villiger oxidation of *rac*-**1** in a recycle reactor: (○) ketone concentration in feed and (● in the fermenter.

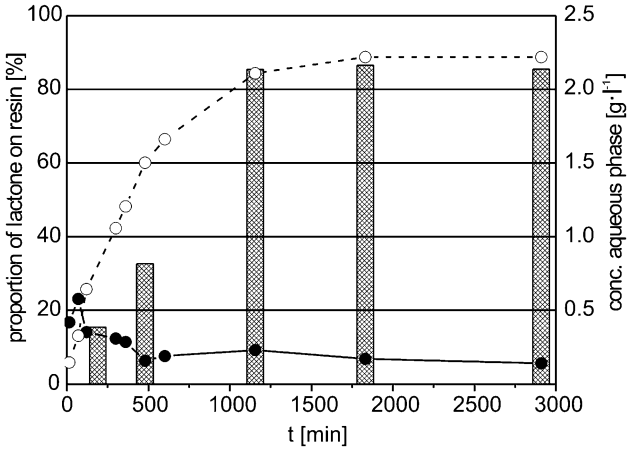
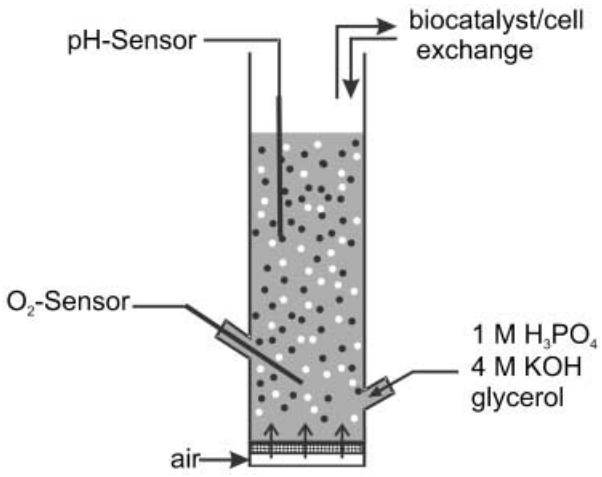


Figure 5. Baeyer–Villiger oxidation of 25 g of **1** in a 1-L recycle reactor (without change of biocatalyst). ▨ proportion of readsorbed combined lactones extracted from a resin sample, (●) ketone concentration and (○) lactone concentration of the cell broth inside the fermenter.

Use of a Bubble Column Reactor

A third interesting type of bioreactor could be a so-called "bubble column" set-up, where aeration takes place through a sparger from the bottom of the column.[18] Thus, mixing of the liquid phase and dispersion of the resin is achieved by the rising air bubbles only. Bubble size and therefore air-liquid interface and mass transfer can be controlled by choosing the appropriate sparger porosity. In general, further mass transfer enhancement is due to elevated oxygen solubility in the bottom region of the column and to longer residence times of the bubbles in the reactor.

In order to achieve our experiments, we designed a 1-L laboratory scale bubble column (Scheme 4) equipped with an air supply of sintered glass, which produces fine air bubbles, at the bottom of the vessel. Although the effect of elevated hydrostatic pressure on oxygen



Scheme 4. Scheme of a "bubble column" set-up.

solubility is surely of no significance at a small scale, an improvement of aeration by increasing the surface-to-volume ratio can be expected. The chosen air flow of 3.3 vvm was able to distribute the adsorbent resin uniformly in the cell broth all over the column.

The results of the BV oxidation of *rac*-**1** in this bioreactor are displayed in Figure 6. At the end of the first cycle, the proportion of lactones on the resin was already higher than 80%. Decantation of the resin (by stopping the air flow) and replacement of the biocatalyst led to completion of the reaction within a few more hours of a second cycle. Ketone **1** and combined lactones (**2** and **3**) concentration in the liquid phase developed as in the conventional and recycle fermenters. Although biooxidation was faster, adsorption or desorption did not become rate-limiting. CHMO activity of the cells was observed during approximately 24 hours, which is comparable to the experiments with the other reactor types.

Obviously, biotransformation performed better in this set-up. This was most probably due to the intensified gas-liquid mass transfer as expressed by the DOT of the cell broth (Figure 9). Even if DOT did not exceed 15% during the time cells were active, it never went down to zero like in the conventional fermenter experiment. Therefore it appeared that oxygenation of the cell broth was improved. However, shortage of oxygen still remained a limitation.

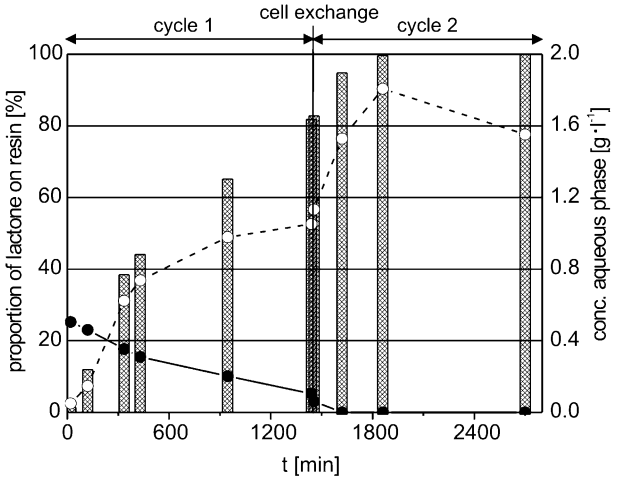


Figure 6. Baeyer–Villiger oxidation of 25 g of *rac*-**1** in the 1-L bubble column over two cycles: Exchange of biocatalyst after 24 hours. ▨ proportion of re-adsorbed combined lactones (**2** and **3**) extracted from a resin sample, (●) ketone concentration and (○) lactone concentration of the cell broth.

Comparison of the Three Types of Bioreactors

Glycerol Consumption, pH Regulation and Oxygen Profiles

Besides DOT or ketone and combined lactone concentration in the cell broth and on the adsorbent resin, other parameters have been determined in the different reactors. These were: consumption of KOH solution, of phosphoric acid, of glycerol, and variation of glycerol concentration. Preliminary to our experiments, glycerol consumption was roughly estimated to 0.3 g g^{-1} dry weight h^{-1} which corresponds to a glycerol feed of 1.9 g h^{-1} for 6.4 g L^{-1} dry weight of cells. Then, the initial glycerol concentration was adjusted to 10 g L^{-1} before starting biotransformation. Actual glycerol concentration was verified using an enzymatic UV assay from reactor samples and the glycerol feed was adjusted according to these results. This method worked satisfactorily in all the three reactors, as shown for the recycle reactor (Figure 7) and for the bubble column (Figure 8). The dosing of glycerol proved to be necessary because for experiments conducted without supply of glycerol the reaction stopped after two or three hours.

Regulation of pH in the cell broth interestingly led to a remarkable difference in the recycle reactor as compared to the other two reactors. Figures 7 and 8 show the consumption of potassium hydroxide solution or phosphoric acid. In the conventional fermenter (not shown here) and in the bubble column, only base was consumed, indicating a continuous production of acid by the cells. In contrast to this, base consumption stopped after one hour in the recycle reactor. The cell broth tended to become basic, thus starting to need addition of phosphoric acid to maintain the pH at 7.2. Only after 20 hours (when the CHMO activity stops down) did this tendency change again. This behaviour can possibly be explained by mechanical stress, since cells are exposed to high shear when being passed through the peristaltic pump and the adsorbent column.

Comparison of the dissolved oxygen profiles of the three reactors indicated a clear oxygen limitation for all the alternatives (Figure 9). A rough estimation of the apparent biocatalyst activity in the different reactors during the first three hours of biotransformation were: 15, 18 and 35 U g dry weight^{-1} for, respectively, the conventional fermenter, the recycle reactor and the bubble column reactor, demonstrating a clear advantage for the bubble column. These results have, however, to be compared to the whole cell activity (i.e., 60–100 U g dry weight^{-1}) determined in the shake flask assay and therefore point out that, although the bubble column performs noticeably better than the set-ups including a fermenter, there is still some space for further optimisation.

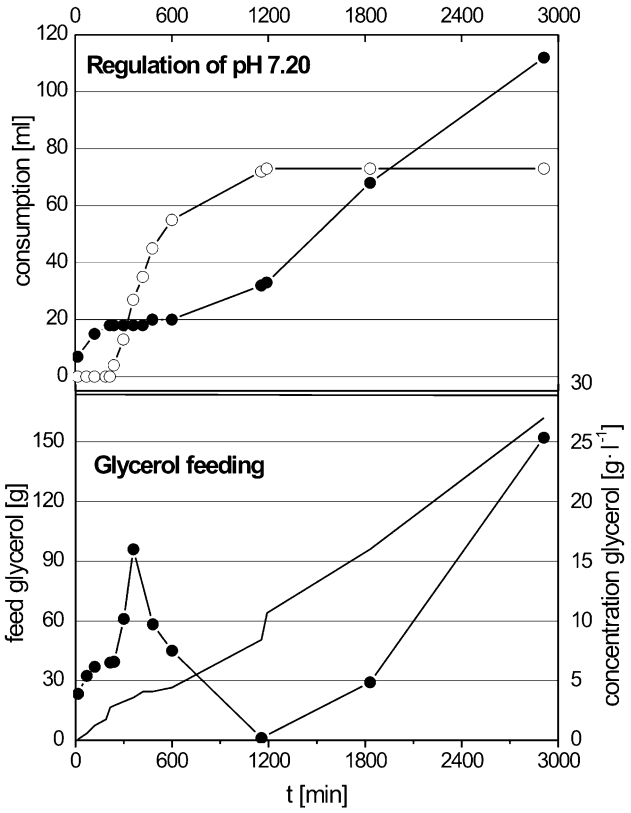


Figure 7. Baeyer–Villiger oxidation of *rac*-**1** in the recycle reactor. *Upper diagram:* Regulation at pH 7.2 with –●– 1 M H_3PO_4 [mL] and –●– 4 M KOH [mL]. *Lower diagram:* Glycerol availability. –●– glycerol concentration in cell broth [g L^{-1}], – glycerol feed [g].

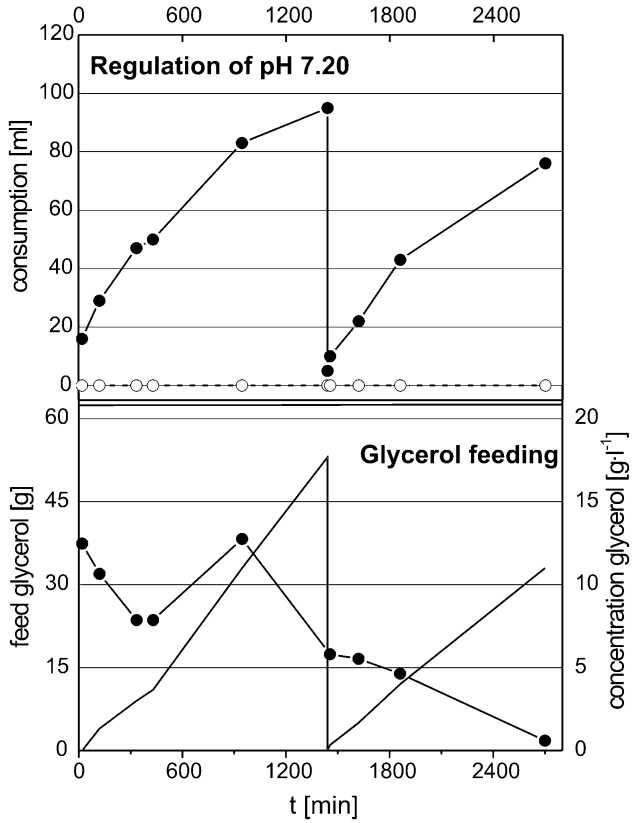


Figure 8. Baeyer–Villiger oxidation of *rac*-**1** in the 1-L bubble column. *Upper diagram*: Regulation at pH 7.2 with –●– 1 M H_3PO_4 [ml] and –●– 4 M KOH [ml]. *Lower diagram*: Glycerol availability. –●– glycerol concentration in cell broth [g L^{-1}], – glycerol feed [g].

Comparison of Yields and Product Quality

After the bioconversion, continuous extraction of resin and liquid phase yielded a yellow or orange oil. Bulb-to-bulb distillation of the crude product removed impurities from the cell broth which also adsorb on the resin during bioconversion and was efficient enough to give a colourless mixture only composed of the two regioisomeric lactones **2** and **3**. A further purification could be carried out by column chromatography or fine distillation to separate these lactones.[14] The BV oxidation of **1** in the fermenter gave 21.8 g of combined lactones or 76% yield after distillation (GC analysis). In the recycle reactor, a yield of 59% (16.8 g) combined lactones (GC analysis of the distilled product) was obtained. This lower yield was due to the fact that the bioconversion was stopped before complete substrate consumption. The proportion of ketone on the resin being still 15% at the end, the yield (with respect to consumed ketone) was 70%. Since the substrate from the bubble column was completely converted, no residual ketone was found in the crude extract. After distillation, 24.3 g of combined lactones showing a purity of 96%, were obtained. This corresponded to a total yield of 84%. Noteworthy, the ee

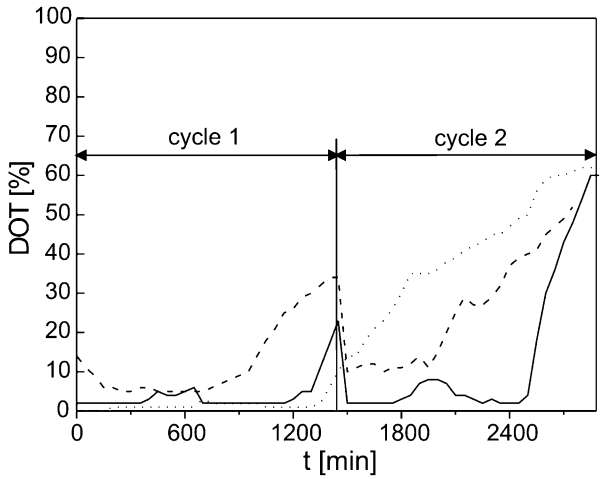


Figure 9. Dissolved oxygen profiles in the different reactor in – fermenter, ····recycle reactor, – – – bubble column.

of each one of the two lactones **2** and **3**, was very high (both >98% since the respective other enantiomer of each lactone was not detected by chiral GC), which is another utmost important point as far as product quality is concerned. In particular, it is to be stressed that,

probably due to the low aqueous phase concentration of **1** in these bioreactors, the ee of the "expected" lactone **2** was always excellent, suggesting that the CHMO enzyme is working at a sufficiently low ketone concentration.[22]

Process Efficiency

Some additional important advantages of the above described processes are: a) the simplicity of their set-up, b) the fact that they allow total use of the substrate and efficient recovery of the product(s). These two points certainly are important features as far as ease of purification and cost efficiency are concerned, in particular for industrial level implementation.

As far as simplicity is concerned, it is to be emphasised that biocatalyst production on one hand, and biotransformation on the other, were conducted in two separate vessels, i.e., a normal fermenter to control and optimise the cell growth and, in a second step, one of the three bioreactors described above. We indeed have observed that the grown *E. coli* cells could be kept at 4 °C (in the refrigerator) without important loss of Baeyer–Villigerase activity (less than 30% loss over 7 days). This is particularly interesting since it allows us to use a repeated batch strategy, and therefore to drive the bioconversion to completion. Further insights in the stability and status of the whole-cell biocatalyst are important to improve process efficiency.

The interesting cost efficiency is linked to the fact that, on the one hand, culture of a (class 1) *E. coli* strain is easy and rather cheap and, on the other hand, that the "protection" of the (expensive) substrate and product within the adsorbent resin avoids important loss of material. Also, the possibility to easily separate the substrate and/or product from the cell culture by simple decantation, filtration and continuous extraction of the resin using a minimal volume of solvent is an additional practical benefit of this "two-in-one" *in situ* SFPR strategy. Last but not least, the ability offered by this approach to run bioconversion on 25 g (0.23 M) in a 1-L vessel favourably compares with the previously described fed batch oxidation of **1**, were much lower quantities of substrate could be transformed per litre of initial cell broth.[14] Owing to the above-described bioconversion profile of the bubble column reactor, the volumetric productivity was about 1 g L^{-1} h^{-1} (7.7 mmol L^{-1} h^{-1}) which corresponds to 20 U g dry $weight^{-1}$. The maximum activity of the cells in this device being maximum over the first 24 hours period, it also can be extrapolated that transforming one kilogram of *rac*-**1** would only need about 50 L of cell culture.

Conclusion

We describe in this work the set-up of an efficient preparative scale process for achieving the asymmetric Baeyer–Villiger oxidation using a biocatalytic (whole cells) strategy, based on the simultaneous use of a recombinant *E. coli* strain and an adsorbent resin. The latter allows us to carry out a "two-in-one" procedure combining *in situ* substrate feeding and product removal (SFPR). Three types of bioreactors, i.e., a conventional reactor, a recycle reactor and a bubble column reactor, were studied and compared. The best one proved to be the bubble column reactor, where 25 g (0.23 M) of *rac*-bicyclo[3.2.0]hept-2-en-6-one (**1**) could be totally transformed using a one-litre vessel (and an additional biocatalytic cycle to drive the reaction to completion), with a volumetric productivity of about 1 g L^{-1} h^{-1} (7.7 mmol L^{-1} h^{-1}). This led to the nearly exclusive production of the two corresponding regioisomeric lactones **2** and **3**, which were both obtained in excellent enantiomeric purity (ee > 98%) and high yield.

To the best of our knowledge, this strategy represents up to now the best example of a (highly productive) preparative scale asymmetric Baeyer–Villiger oxidation. The oxygen limitation being one of the obvious remaining limitations of this process, an improvement of this factor could certainly afford even higher productivity. Studies aimed to scale up this new methodology to larger pilot scale quantities and to apply it to other types of substrates are presently under way in our laboratories.

Experimental Section

Chemicals

The media components for fermentation [soybean peptone (70178), yeast extract (70161), glycerol, ampicilline sodium salt, L-(+)-arabinose (99%)] as well as racemic bicycle[3.3.0]-hept-2-en-6-one (**1**; 98%) were obtained from Fluka. Polymer resins [Amberlite (Rohm & Haas), Optipore (Dow), Sepabeads (Mitsubishi) and Lewatit (Bayer)] were obtained from Fluka or Supelco. Active charcoal (05112), active charcoal "Norit RO 0.8" (22875) and active charcoal "Norit Darco" (96831) were donated by Fluka.

Adsorption Equilibria of Bicyclo[3.2.0]hept-2-en-6-one (1)

The performance of different adsorbent materials (active charcoals and polymer resins) was evaluated by tracing their respective adsorption isotherms. Varying quantities of ketone and the mass of adsorbent corresponding to a dry weight of 50 mg are equilibrated in 4 mL of distilled water at 25 °C for 24 hours. The equilibrium concentration in the aqueous phase of each sample was then determined by extraction and GC

analysis. Because all adsorbents are delivered with a water content that changed considerably from batch to batch and over storage time, the humidity of the materials was determined first by drying a known quantity of the adsorbent at 60 °C over night. Adsorbents are used wet without further treatment, their mass is recalculated according to the dry weight which is used in the definition of the load X (see above).

Fermentation of *E. coli* TOP10 (pQR239)

The fermentation procedure corresponded in large parts to the one given by Doig et al.[21] for this strain. The growth medium used in precultures and fermentation consists of 10 g L^{-1} glycerol, 10 g L^{-1} yeast extract, 10 g L^{-1} soybean peptone and 10 g L^{-1} NaCl. It was autoclaved at 115 °C for 20 min. Immediately before inoculation 0.1 g L^{-1} ampicilline was added as an aqueous solution which was sterilized by filtration (0.2 μm).

Inoculum cells were grown in two steps from a glycerol stock culture of *E. coli* TOP10 (pQR239) kept at − 80 °C. The first preculture has a volume of 10 mL (in a 100-mL shake flask) and was inoculated with 300 μL of stock culture. After 6 hours growth at 37 °C, 300 μL cells were inoculated into the second preculture of 6 × 75 mL in 500-mL shake flasks. These were grown over night at 37 °C and inoculated into 5 L of growth medium in a stirred 10-L fermenter (Bioflo 3000, New Brunswick Scientific, N. Y., USA) equipped with pH and oxygen probes. Growth conditions in the fermenter were pH 7.2 adjusted with 1 M KOH and 1 M H_3PO_4 , agitation speed of 500 rpm and an aeration of 1.1 vvm. The cells grew within 5 – 6 hours and their induction took place in the late log phase by adding L-(+)-arabinose in a concentration of 1 g L^{-1} to the fermenter. At this time, the dissolved oxygen tension (DOT) in the medium was near zero. One hour after induction, cells were harvested in their medium and stored at 4 °C for further use (3 – 5 days without significant loss of activity if glycerol was not depleted). The BVase activities (against **1**) were about 60 – 100 U g dry weight^{-1}.

Analytics

CHMO activity assay

Subsequent to fermentation, whole cell CHMO activity was measured in a shake flask assay. 10 mL of cells directly from the fermenter were mixed with 10 mL of phosphate buffer (500 mM) at pH 7.2 in a 250-mL shake flask at 30 °C. After addition of a 50 g L^{-1} (±)-bicyclo[3.2.0]hept-2-en-6-one (**1**) solution in ethanol to attain an initial ketone concentration of 0.5 g L^{-1}, aliquots were taken at regular time intervals, extracted with ethyl acetate and analyzed by GC. The combined lactone formation is monitored for one hour and from its initial velocity, specific whole cell activity is calculated in U g dry weight^{-1}.

Gas chromatography

Concentrations of bicyclo[3.2.0]hept-2-en-6-one (**1**) and its corresponding lactones **2** and **3** were determined by extraction of a sample of the aqueous phase with ethyl acetate containing 0.5 g L^{-1} undecane as internal standard. Samples of adsorbent material were extracted with ethyl acetate only. The organic phase was then analyzed at 110 °C on a Shimadzu GC 14-A equipped with a silica column (Optima-5, Macherey-Nagel, No. 726099.30) and an FID. The enantiomeric excess of the lactones was determined on a chiral column (DMePeBetaCDX (0V1701), Mega, Legnano, Italy) at 110 °C.

Determination of glycerol concentration

Glycerol concentration in the culture medium was checked using an enzymatic UV assay (Scil Enzytec™ Glycerol ID-No. 1 002 809).

Biotransformation Procedure

Loading of the adsorbent with substrate

The adsorbent was loaded with ketone **1** separately before biotransformation. Dowex Optipore L-493 was used as received with a current degree of humidity of 52%. 25 g (0.23 mol) of (±)-bicyclo[3.2.0]hept-2-en-6-one (**1**), the corresponding amount of humid Optipore L-493 and 200 mL of culture medium were shaken gently during 1 hour to equilibrate. The load with respect to dry adsorbent resin was adjusted to approx. 0.3 and the aqueous equilibrium ketone concentration to 0.5 g L^{-1} in case of fermenter and bubble column, whereas values for the recycle reactor were 0.5 and 2.0 g L^{-1}, respectively.

Biotransformation

Of 1 L of cell broth (stored at 4 °C), 200 mL were centrifuged and the supernatant was discarded to avoid dilution by adding the liquid from the loading procedure. The pellet was resuspended in the remaining broth which was then poured into the reactor. The cells were held for half an hour at a temperature of 37 °C and an aeration of 3.3 vvm. The pH was regulated at 7.2 with 4 M KOH and 1 M H_3PO_4 and a continuous glycerol feeding was installed. Before starting biotransformation, glycerol concentration was adjusted to 10 g L^{-1} and 2 mL antifoam (Pluronic PE 1800 – BASF) were added. During biotransformation, the glycerol concentration was verified regularly and continuous addition was controlled manually. Addition of antifoam was repeated if necessary.

If cells were exhausted and deactivated before complete conversion of ketone, a second cycle of biotransformation could be run by simply separating and replacing the cell broth with fresh biocatalyst from the stock at 4 °C.

Conventional fermenter: The fermenter was used was a stirred 1.5-L model of Setric Génie Industriel (Toulouse, France). Impeller speed was adjusted to 700 min^{-1}. The preloaded resin was just added to the cell broth to start biotransformation. Cell concentration in this experiment was 6.4 g L^{-1} dry weight and specific whole cell activity was 96 U g dry weight^{-1}.

Recycle bioreactor: In this reactor, the fermenter was connected to a preparative chromatography column (Modu-

line, Amicon Wright Ltd., England). The substrate loaded resin was placed in this column as a fixed bed. The liquid phase with the cells was recycled through the fixed bed by means of a peristaltic pump at 100 mL/min. Inlet and outlet were protected by a 100 μm filtration tissue keeping the resin inside but letting cell broth pass easily. The impeller speed in the fermenter was 700 min^{-1}. Biocatalyst exchange was especially convenient in this arrangement with the resin retained in the column. In this experiment, cell concentration was 10 g L^{-1} dry weight and specific whole cell activity was 90 U g dry $weight^{-1}$.

Bubble column bioreactor: A scheme of the bubble column bioreactor is shown in Figure 4. Aeration took place through a sparger in form of a porous sintered glass in the bottom of the column. No additional mixing device was needed, adsorbent resin and cells were dispersed only by the rising air bubbles. The column had an inner diameter of 65 mm and a length of 300 mm. The tip of the oxygen probe was situated 34 mm above the sparger. To avoid contamination with other microorganisms, the column was closed by a top cover with acid, base and glycerol entries and air outlet. The culture, and therefore cell concentration and specific whole cell activity, was the same as indicated for the 1.5-L fermenter.

Product Isolation and Purification

The experimental procedure used for isolation of the biotransformation products was identical whatever the type of reactor used. In a typical experiment, the product was continuously extracted from the liquid phase by CH_2Cl_2 (for 24 – 48 hours). The resin was extracted by ethyl acetate in a Soxhlet extractor. The organic phase was dried over $MgSO_4$ and concentrated. The crude product was simply purified by bulb-to-bulb distillation to give a "pure mixture" of lactones **2** and **3**, obtained as a colourless oil.

Acknowledgements

This work was funded by the Project QLK3-CT2001 – 00403 "BIOMEX" as part of European Community Fifth Framework Programme and supported by the Swiss Federal Office for Education and Science (BBW).

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