Organic Process Research & Development **2008,** *12,* 660–665

The First 200-L Scale Asymmetric Baeyer−Villiger Oxidation Using a Whole-Cell Biocatalyst

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Abstract:

Biocatalytic Baeyer−Villiger oxidations using oxygen as an environmentally friendly oxidant in aqueous media have been shown to proceed with excellent stereo- and enantioselectivity for a large number of substrates at laboratory scale. These are good starting boundary conditions for process research and development compared to systems with reactive oxidants and flammable organic solvents. In this paper we discuss some of the considerations required to scale up a whole-cell biocatalytic oxidation from the laboratory to pilot-plant (200 L) scale. Issues for fermentation, bioconversion, and product recovery are discussed, supported by data from pilot-plant and scale-down experimentation. A simple fed-batch approach has been used.

Introduction

There is little doubt that in the synthesis of new optically pure molecules, biocatalysis (whether carried out by an isolated enzyme or by an intact cell, with one or more enzymes of interest overexpressed) has a vital role to play. The exquisite stereocontrolled chemistry that can be carried out by enzymes should make them frequently the catalyst of choice when creating or resolving stereogenic centres. However only a handful of enzyme classes have been used at scale, and the majority of biocatalytic conversions are carried out with isolated hydrolases, resolving a stereogenic center. In recent years much has been done to redress this imbalance with chemists increasingly turning to enzymes for carbon−carbon bond synthesis, and redox conversions. There are numerous examples of catalytic asymmetric oxidation reactions such as heteroatom oxidation, Baeyer−Villiger oxidation, epoxidation, mono- and dihydroxylation at nonactivated carbon atoms, which have been catalyzed by oxygenase enzymes in whole-cell systems at laboratory scale. Nevertheless for industrial implementation it will be essential for the necessary scale-up and process research and development to be successfully completed as well.[1] In this paper, we will discuss the scale-up considerations required to take a whole-cell biocatalyst from laboratory to pilot-scale operation at 200 L, using Baeyer−Villiger oxidation as an example.

The Baeyer−Villiger oxidation is an important ketone transformation in many syntheses[2] and is generally carried out using oxidants in stoichiometric quantities, which at large-scale frequently cause a problem to pass successfully current safety, health and environmental (SHE) risk assessments. The simultaneous presence of stoichiometric amounts of reactive oxidants and flammable organic solvents thereby represents operating conditions which require substantial safety precautions and have the disadvantage of generating stoichiometric amounts of waste. Modern catalytic and asymmetric versions of this key transformation therefore continue to be of major interest for selective and ecoefficient Baeyer−Villiger oxidations, and in recent years inorganic[3,4] and organic catalysts[5] as well as more environmentally friendly oxidants[6] have been developed. However, it is the combined use of biocatalysis with molecular oxygen as oxidant which shows both selectivity and practical safety, health, and environmental advantages.[7]

Baeyer−Villiger monooxygenases (BVMOs) are a group of enzymes that can catalyse the stereoselective oxidation of linear and cyclic ketones, yielding esters and lactones, respectively.[8]

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Published on Web 07/18/2008
10.1021/op800046t CCC: $40.75

Since enantiomerically pure esters and lactones are useful intermediates in the production of pharmaceuticals and agrochemicals and are difficult to obtain directly by chemical Baeyer−Villiger oxidations, the development of a scalable BVMO-catalysed process[9–14] is of major importance. One of the best characterised enzymes of this group is cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 which can oxidise a wide range of ketones.[15] However, large-scale bioprocesses using CHMO are hampered by several limitations. Most notable are (1) the pathogenic classification of the wild-type host, (2) complexities in the growth and expression of CHMO activity, (3) the presence of a lactone hydrolase, and (4) the absolute requirement for stoichiometric quantities of the cofactor NADPH.[15] The combination of these issues makes use of the wild-type whole-cell catalyst unappealing, and use of the isolated enzyme complex unattractive too since an NADPH recycling system is obligatory on account of its high cost.

In an attempt to solve these problems, with our collaborators, we[16–18] and others,[19–25] have constructed and tested several different recombinant whole-cell biocatalysts expressing CHMO, and in this way circumvented all of the above problems. Stewart and co-workers described the creation of a recombinant *Saccharomyces cerevisiae*[19,20] and latterly have also cloned and overexpressed CHMO in *Escherichia coli*.[21,22] Both these strains have been shown to be versatile biocatalysts, catalysing a wide range of Baeyer−Villiger oxidations as well as stereoselective sulfide oxidations. However, detailed kinetic characterisation and subsequent optimisation of the fermentation and biotransformation processes using these strains has not yet been carried out. Furthermore, although in most cases these recombinant biocatalysts showed good yields of product on reactant, the initial inventory of reactant and thus final product concentration was rather low at about 1 $g \cdot L^{-1}$, and certainly not commercial. In addition, in some cases there was evidence of unwanted side reactions occurring, for example ketone reduction.[19]

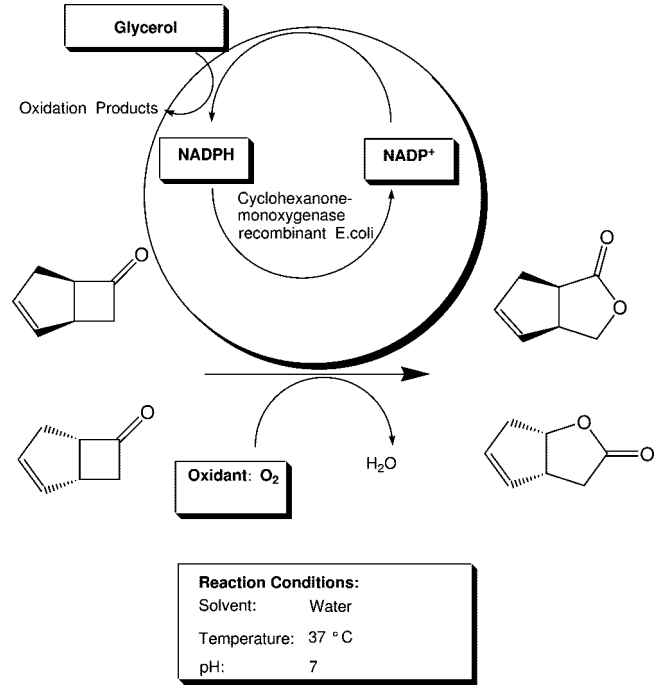

Figure 1. **Baeyer−Villiger monooxygenase-catalysed oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one to an equimolar mixture of (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one and (−)-(1*S*,5*R*)-2-oxabicyclo-[3.3.0]-oct-6-en-3-one.**

Previously, with our collaborators we have cloned and overexpressed CHMO into *Escherichia coli* TOP10 [pQR239] and with this novel strain demonstrated a pilot-scale (300 L) fermentation process yielding between 5 and 6 $gdcw \cdot L^{-1}$ and exhibiting a specific whole-cell activity of nearly 55 $\mu mol \cdot min^{-1} \cdot g^{-1}$ (0.41 $g \cdot g^{-1} \cdot h^{-1}$) for the oxidation of bicyclo[3.2.0]hept-2-en-6-one[16,17] (Figure 1). The key kinetic characteristics of this biocatalyst for this conversion were severe reactant and product inhibition. Maximum specific activity occurred at a ketone concentration of 0.2 $g \cdot L^{-1}$, whilst at 4 $g \cdot L^{-1}$ the activity was reduced by 90%. Furthermore, at a product concentration of 4.5 to 5 $g \cdot L^{-1}$, the specific activity fell to zero. Nevertheless, by employing a ketone feeding strategy, a final product concentration of 3.5 $g \cdot L^{-1}$ could be achieved at moderate scale (55 L). The lactones produced had an enantiomeric excess of >95% and the overall process yield of product on reactant was 85% and of product on biocatalyst was 0.7 $g \cdot g^{-1}$. It was shown that this process was, after implementation of substrate feeding, limited by product inhibition.[18]

In order to overcome these problems, strategies to control both the substrate and the product concentrations as well as mass transfer into and out of the cell are required. Some solutions, which have been tested specifically for the Baeyer−Villiger oxidation, include the use of a water-immiscible organic phase to serve as a substrate reservoir and product sink up to a concentration of 5 $g \cdot L^{-1}$ [26] and a selective adsorbent resin as a reservoir for 20 $g \cdot L^{-1}$ of the reactant and as a sink for the lactone produced.[27–29] Although these techniques proved very

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Table 1. **Storage stability of cells**

storage	specific activity	observed reaction rate
fresh cells	1040 $U \cdot g^{-1}$	0.67 $g \cdot L^{-1} \cdot h^{-1}$
overnight at 4 °C	940 $U \cdot g^{-1}$	0.66 $g \cdot L^{-1} \cdot h^{-1}$
overnight at 20 °C	772 $U \cdot g^{-1}$	0.71 $g \cdot L^{-1} \cdot h^{-1}$

valuable for achieving high concentrations for the biocatalytic Baeyer−Villiger oxidation, large-scale boundary conditions and the optimal interface to subsequent chemical reaction steps might favour alternative process concepts for the biocatalytic Baeyer−Villiger reaction step.[30] Hence, alongside these multiphasic solutions, we have also examined a simple controlled substrate addition option, and in this paper we report on the scale-up of such an approach in the pilot-plant to 200 L.

Results

Catalyst Preparation and Scheduling. Processes using whole-cell catalysts have the added issue of integrating catalyst preparation with the bioconversion step. Conventional wisdom holds that a number of advantages can be realized by separating fermentation (catalyst production) from bioconversion (reactant conversion). Optimisation of each operation and avoiding compromising conditions may give benefits to the catalyst and product yield.[31,32] However, despite the benefits of such an approach, the scheduling of operations becomes an issue. For example at the end of the fermentation it may be necessary to proceed directly with the bioconversion. At scale, the conversion may take 6−8 h, rendering the entire process unworkable in a single day. Consequently, the need for intermediate storage would be very useful (enabling operation in two standard operating days), and therefore we examined this with biocatalyst held at 4 and 20 °C. The results are shown in Table 1. It is clear that the specific activity falls with storage. However, the specific activity is a measure of the catalytic potential of the cells (i.e., all the available protein) since it is measured by depletion of NADPH in a standard cyclohexanone conversion. Interestingly, in all cases despite changing specific activity they each have an observed activity which is very similar and agrees with previously published results. We believe the observed activity is limited by supply of this substrate into the cells as we have previously reported.[33] Interestingly, this observed activity appears in many experiments to be the maximum rate obtainable in the absence of oxygen limitation. The results therefore indicate we can use the cells with overnight storage without any decrease in observed activity. This makes the scheduling far easier with fermentation on Day 1 followed by bioconversion on Day 2. The other benefit of separating fermentation from bioconversion is that it enables the resuspension of the cells both at a different concentration and in different media. In previous work the influence of cell and oxygen concentrations on the biocatalytic Baeyer−Villiger oxidation under various experimental conditions has been described.[34,35] Of particular interest is the way process metrics change with catalyst concentration. For example increasing the catalyst concentration does not always give the same specific activity since the oxygen is required first for cell maintenance followed by that required for the conversion. The optimum cell concentration therefore depends on the oxygen supply. In a systematic study we have evaluated the effect of both catalyst concentration and also enhanced oxygen supply.[36] Hence the intriguing possibility of resuspension in non-fermentation media could bring considerable improvements in specific activity but could also have benefits for downstream processing. In separate experiments bioconversions using the same batch of cells were run in water, fermentation media and NaCl solution with the aim of establishing an alternative to fermentation media. The results (shown in Table 2) indicate that there are few problems with changing media. All subsequent experiments were carried

Table 2. **Effect of re-suspension of cells in alternative diluents**

diluent	observed reaction rate
medium	0.78 $g \cdot L^{-1} \cdot h^{-1}$
phosphate buffer	1.13 $g \cdot L^{-1} \cdot h^{-1}$
water with 10 $g \cdot L^{-1}$ NaCl	1.22 $g \cdot L^{-1} \cdot h^{-1}$
water	1.29 $g \cdot L^{-1} \cdot h^{-1}$

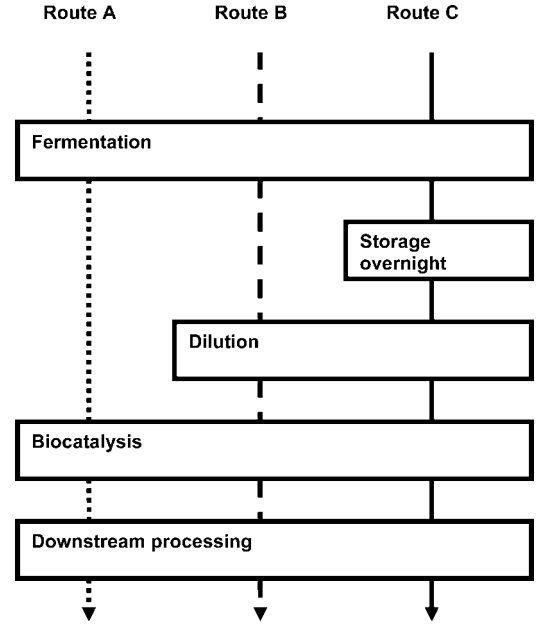

Figure 2. **Scheduling options for the Baeyer−Villiger monooxygenase-catalyzed bioconversion. Route A (dotted line): the fermentation is followed directly by the bioconversion step, taking a minimum of 12 h. Route B (dashed line): the bioconversion step is optimized for oxygen transfer and cell concentration by diluting the fermentation, taking additional time. Route C (solid line): The fermentation and dilution/bioconversion steps are separated by a storage step, allowing the fermentation to be carried out on a first standard working day followed by bioconversion on a second standard working day.**

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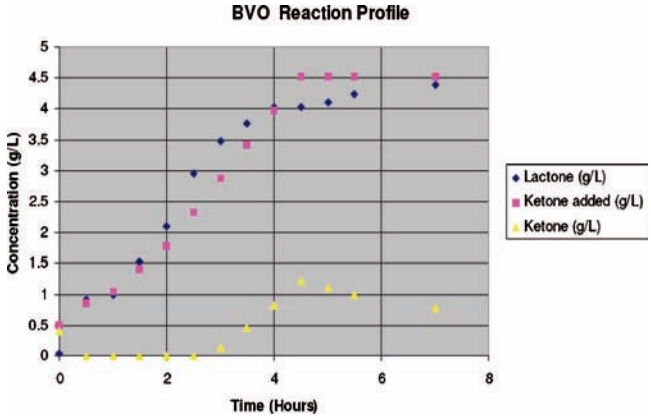

Figure 3. **Two-hundred liter scale fed-batch bioconversion.**

out in water with NaCl. The addition of salt appears to improve the downstream processing. The possible flowsheets for the preparation of catalyst at scale are shown in Figure 2.

Bioconversion and Substrate Feeding. It is well established that, aside from product inhibition, the conversion of bicyclo[3.2.0]hept-2-en-6-one is primarily limited by the need to control the reactant in the vicinity of the *E.coli* cells.[37] At a small scale low concentrations can be used in batch mode. But as the scale of reactor increases, it is increasingly important to produce a high enough concentration of product in order to limit the scale of the downstream process. Two alternative routes can be followed. The simpler route is to feed the reactant in concentrated form, with the proviso that mixing of the substrate must be easy (which itself will be a function of scale). The second method is to use an auxiliary phase (such as an organic solvent or a resin) to supply and potentially also remove the product. The advantage of the latter method is that some form of *in situ* product removal (ISPR) can also be effected as the reaction proceeds,[38] eliminating product inhibition problems. The use of resins has been shown to be highly effective for the oxidation of bicyclo [3.2.0]hept-2-en-6-one to give a 20 $g \cdot L^{-1}$ product concentration[27,28] via control of substrate and product concentrations. Nevertheless the need to select the resin makes the former route for reactant feeding particularly attractive for rapid scale-up. Figure 3 shows the concentration-time profile for the oxidation of bicyclo[3.2.0]hept-2-en-6-one to its two regio-isomeric chiral lactones (see Figure 1) using *E. coli*. In this conversion we used a biocatalyst concentration of 1.5 $g \cdot L^{-1}$ in order to avoid oxygen limitation.[34] While the control of the substrate concentration in solution is difficult, the reaction proceeds smoothly and 4.5 $g \cdot L^{-1}$ of product has been made at a 200-L scale. To the best of our knowledge, this is the first such operation of the whole-cell BVMO-catalysed conversion of a cyclic ketone at this scale. Process improvements are possible via online measurement and feedback to control the substrate addition rate. The extent of mixing will require careful investigation as the scale is increased further.

Product Recovery. The steps for the product recovery after the biocatalytic reaction are summarized in the flowsheet in Figure 4. After completion of the large-scale biocatalytic

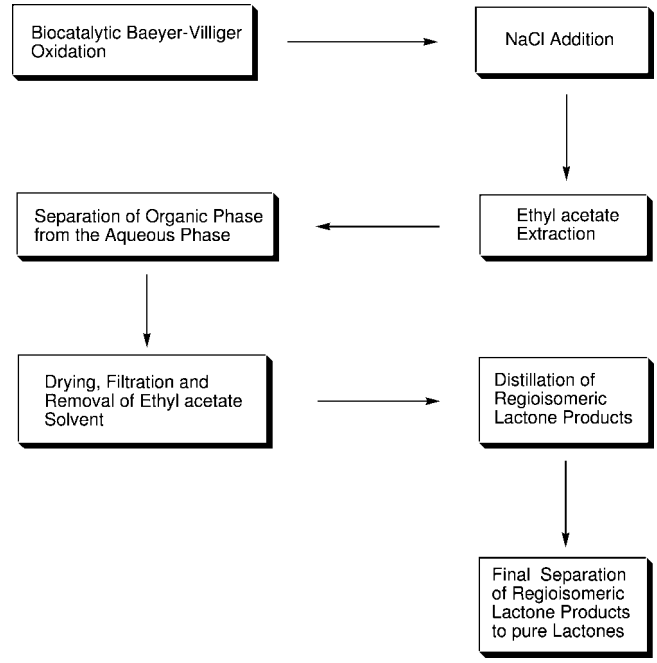

Figure 4. **Product recovery flowsheet.**

Table 3. **Comparative data between biocatalytic reactions at 1.5-, 55-, and 200-L scale**

scale L	biocatalyst concentration ($g \cdot L^{-1}$)	maximum activity ($g \cdot L^{-1} \cdot h^{-1}$)	ketone feed rate ($g \cdot L^{-1} \cdot h^{-1}$)	final concentration ($g \cdot L^{-1}$)
1.5	5.0	2.1	0.8−3.2	3.5
55	5.0	2.1	1.0	3.9
200	1.5	0.6	0.6−1.1	4.5

Baeyer−Villiger reaction, the conditions for the most efficient downstream processing were established with the real reaction mixtures containing both lactones and cells. The separation of the aqueous and the organic phase can be accelerated by further sodium chloride addition, and the product-containing ethyl acetate phase was easily recovered. Removal of the solvent and distillation followed standard scalable procedures and yielded a 50/50 mixture of the enantiopure regioisomers (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0] oct-6-en-2-one and (−)-(1*S*,5*R*)-2-oxabicyclo-[3.3.0]-oct-6-en-3-one, which could subsequently be separated by preparative chromatography.[17]

Discussion

A comparison of the data from conversions at 1.5-, 50-, and 200-L scales indicates that the process is easily scalable (Table 3). The cell and oxygen concentrations can be selected within the windows of operation, avoiding oxygen limitation at a given cell concentration.[34] The process itself is fairly robust with respect to ketone accumulation, but it is certainly best to control the ketone addition in the process by GC-analysis of the online concentration in the reaction mixture. A decisive influence on the downstream processing comes from the complete conversion of the ketone. The absence of ketone in the 50/50 mixture of enantiopure lactone regioisomers simplifies the chromatographic separation. Consequently finishing the feeding ahead of the completion of the reaction is beneficial.

As the process is scaled, two factors may become limiting. First, we expect the dissolution rate of ketone in solution and

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its mixing to become a problem. For this reason moderate feeding rates are required. From our previous work at 1.5 L scale, it is clear that at the highest feeding rate of 3.2 $g \cdot L^{-1} \cdot h^{-1}$ this becomes a problem. Second we can expect oxygen supply to the reactor to become limiting. In previous work we have studied this in some detail and conclude that operation at scale is best at the more modest catalyst concentrations, in order to make best use of the available biocatalytic activity. Technical solutions exist to overcome oxygen supply limitations via increased air flow rate, agitation speed, and suitable sparger design. However, the aim here was to operate the simplest system in order to accelerate scale-up, and therefore modest catalyst concentrations have been used. Overall, this large-scale process design has been shown to work successfully at 200 L, and the simple operation of the biotransformation part of the Baeyer−Villiger reaction in water constitutes a major step forward to improved safety, health, and environmental performance compared with reactive oxidants and flammable solvents.

It is interesting to compare this fed-batch operation at 200 L with the smaller scale (50 L) operation we have conducted at higher concentrations.[27–29] Clearly the 50-L conversion is more efficient (on a volumetric basis) although there are extra costs associated with resin use. Dependent upon the recycle of the resin this process may or may not prove economically preferable to the 200-L scale process. Ultimately in this case the addition of resin results in a 4-fold increase in concentration prior to the downstream process and a smaller reaction vessel. However, this is offset not only by the cost of the resin but also the effort required to screen suitable resins and evaluate resin recycle. Clearly evaluation is on a case-by-case basis, but for many reactions for the time being the simpler technology is quicker to implement and may therefore prove advantageous.

Conclusion

In summary, in this paper we have presented data on the first BVMO process to be operated at 200-L scale. Operation at this scale necessitated the separation of biocatalyst production from reaction, and the intermediate storage and resuspension of the cells has been examined. We can conclude

(1) The process has been operated successfully at scale via the implementation of a conservative approach to substrate feeding and cell concentration, ensuring that limitation at scale does not occur due to either ketone dissolution and mixing or oxygen supply.

(2) Product recovery has been successfully effected via extraction. It is interesting that the use of resuspended cells in water with salt leads to a significant improvement in phase separation, resulting in a higher final product yield.

(3) The fed-batch process produces merely 4.5 $g \cdot L^{-1}$ of product but is based on a simple and scalable methodology ensuring process implementation. In the paper we have presented the minimum set of experiments needed to ensure successful scale-up for this type of conversion.

(4) For the future, scalable methods for the separation of the two regioisomers to recover product will be required. Recent work has focussed on new technologies such as simulated moving bed (SMB) chromatography.[39] An alternative approach is to use an optically pure ketone for the bioconversion, placing the separation problem ahead of the reaction.[40] Evaluation of the strategies will be dependent on available separation methods and the particular characteristics of a given substrate and product pair.

Experimental Section

Chemicals. All fermentation media components (soybean peptone (70178), yeast extract (70161), glycerol, ampicillin, L-(+)-arabinose (99%), antifoam PPG 2000), and racemic bicyclo[3.2.0]hept-2-ene-6-one (99.6% purity, 12108) were obtained from Sigma-Aldrich, Buchs, Switzerland.

Analytical. *Gas Chromatography.* Samples from biotransformations were analyzed by GC. Cell broth samples were extracted with ethyl acetate; in the latter case the solvent contained 0.5 $g \cdot L^{-1}$ of dodecane as an internal standard. The organic phase was injected onto a nonpolar DB5 5 μm column (30 m × 0.53 mm) in an HP5890 series II GC instrument (530 GC01) equipped with an FID. The injector temperature as set at 250 °C, and the temperature programme was 50−250 at 5 $°C \cdot min^{-1}$. The results were obtained with a standard integrator.

UV-Assay for CHMO Activity. The CHMO activity was determined by the NADPH consumption rate during oxidation of cyclohexanone. At 340 nm, NADPH has an absorption coefficient of 6.22×10^{-3} $L \cdot \mu mol^{-1} \cdot cm^{-1}$. Cells were resuspended in 50 mM Tris-HCl buffer (pH 9) with 7 $g \cdot L^{-1}$ bovine serum albumin. They were sonicated (5 times for 10 s) on ice. After centrifugation, the CHMO-containing supernatant was separated from the cell debris. Concentrations of reactants in the assay were 0.161 mM NADPH and 0.2 mM cyclohexanone (all in 50 mM TRIS-HCl buffer pH 9). Typically, CHMO was used at a dilution of 10, but could be diluted further for good linearity in case of high activities.

Fermentation of *E. coli* TOP10[pQR 239]. In all fermentations and precultures, the medium composition was 10 $g \cdot L^{-1}$ soybean peptone, 25 $g \cdot L^{-1}$ yeast extract, 12 $g \cdot L^{-1}$ glycerol, 10 $g \cdot L^{-1}$ NaCl, and 100 $mg \cdot L^{-1}$ ampicilin. Precultures were 10% v/v of the total fermentation volume, and they were grown in shake flasks at 37 °C from glycerol stocks overnight. The fermentation took place at 37 °C and pH 7, maintained with 2 M H_3PO_4 and 2 M NaOH solutions. Antifoam PPG 2000 was added as required; the fermenters from Bioengineering AG (Wald, Switzerland) were equipped with foam probes. The cell broth was aerated at 1.8 vvm and stirred at such a rate that oxygen limitation was avoided as long as possible (1000−1500 rpm in 10-L fermenter, 450 rpm in 300-L fermenter). At an OD_{600} of 20−25, CHMO expression was induced by adding 2 $g \cdot L^{-1}$ L-(+)-arabinose. The cells were left for one more hour and then harvested from the fermenter. Their storage stability was good at 4 °C during 3−5 days. Typical CHMO activities were in the range of 400−900 $U \cdot g^{-1}$ dry weight, dependent on fermentation conditions.

Small-Scale Bioconversions. In order to decide whether large volumes of cell broth could be stored between the

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fermentation and the biotransformation phase, the CHMO activity and the observed reaction rate of the system were measured under different storage conditions. The experiments were performed in shake flasks at 37 °C and pH 7 with fresh cells and after storage of the cells overnight (12 h) at 4 °C and at 20 °C. The CHMO-activities were determined by the UV-assay described in the analytical section and the reaction rates for the Baeyer−Villiger oxidation by GC-analysis of lactone formation as a function of reaction time. The results (Table 1) show that the cell broth can be stored overnight at 20 °C in the fermenter.

The second decision to be taken concerned the diluent with which the planned cell concentration of 1.5 $g \cdot L^{-1}$ for the biotransformation phase should be achieved. Therefore, a series of scaled-down fed-batch bioconversions with different diluents (water, fermentation medium, phosphate buffer and water with 10 $g \cdot L^{-1}$ sodium chloride) were performed. The productivities of the Baeyer−Villiger oxidation for a starting ketone concentration of 1 $g \cdot L^{-1}$ under these different biotransformation conditions are shown in Table 2 and have been determined at 400-mL scale in Sixfors mini-fermenters from Infors AG (Bottmingen, Switzerland). These autoclavable fermenters were fully equipped with feed pumps and temperature, oxygen, pH and antifoam probes. The pH electrodes were tailor-made by Hamilton (Bonaduz, Switzerland). In addition, standard oxygen electrodes from Mettler-Toledo (Greifensee, Switzerland) were used. Biotransformations were carried out at 37 °C and pH 7, maintained with 2 M H_3PO_4 and 2 M NaOH solutions. The lactone formation was analyzed as a function of time by GC. The results clearly show that dilution with water or (water with 10 $g \cdot L^{-1}$ NaCl) gives the best productivities for lactone formation. Water with 10 $g \cdot L^{-1}$ NaCl was chosen for all subsequent experiments as the best diluent, because for downstream processing the phase separation time for the aqueous and organic phases following extraction were shorter for the water with 10 $g \cdot L^{-1}$ NaCl than for water alone.

Biotransformation at 200-L Scale. We used a fermenter of 300 L total volume with standard aeration using a ring sparger. The cell concentration of 1.5 $g \cdot L^{-1}$ was achieved by dilution of 37.5 L of cell broth containing 8 $g \cdot L^{-1}$ dry weight from the same fermentation as the experiments described by Hilker and co-workers,[35] with water containing 10 g NaCl per L to yield 200 L of diluted cell broth. Glycerol concentration in the liquid phase was adjusted to 10 $g \cdot L^{-1}$ at the start. The conditions during biotransformation were *T* at 37 °C, pH at 7 with 2 M KOH and 1 M H_3PO_4, a stirrer speed of 450 rpm and an aeration of 1.8 vvm. Antifoam was added when foaming occurred by using the antifoam probe; 900 g of the ketone bicyclo-[3.2.0]hept-2-en-6-one (8.33 mol) was slowly fed with a pump to the cell broth in the fermenter. The ketone at 10 $g \cdot L^{-1}$ was fed to the reactor at two feed rates: 0−2 h at 0.6 $g \cdot L^{-1} \cdot h^{-1}$, and 2−4.5 h at 1.1 $g \cdot L^{-1} \cdot h^{-1}$. The rates were based on the known activity of the cells at the biocatalyst concentration used (0.6 $g \cdot L^{-1} \cdot h^{-1}$ at 1.5 $g \cdot L^{-1}$ of cells.)

Product Isolation. Into 193 kg of the reaction mixture containing the lactone products and cells was dissolved 20 kg sodium chloride by gently stirring at 150 rpm. The extraction was done with 40 L of ethyl acetate, and the phase separation was achieved by adding 10 kg of sodium chloride. The combined organic phases of three ethyl acetate extractions were dried with sodium sulfate and filtered, and the ethyl acetate solvent was removed on a 50-L Rotavap at 10 mbar and 40 °C. The final lactone product was obtained as a clear, brown oil in a yield of 495 g, which was easily decolorized by distillation to give a colourless liquid in quantitative yield.

Product Purification. The separation of the two enantiomerically pure regioisomers by chromatography was achieved by classical column chromatography[17] and by simulated moving bed (SMB) chromatography.[39] Although other separation methods such as fine distillation were considered, the amount of product mixture was not adequate for a high-yield separation at this scale. A Knauer CSEP916 SMB instrument equipped with 12 Eurosphere100 10 μm bead silica columns (2 cm × 25 cm) was used for the SMB-separation. The raffinate and extract streams were monitored on a Gynkotek HPLC-system with a M480 pump, a UVD 320 diode array detector, and a GINA160 autosampler. Both regioisomers were obtained with a purity of 99% and nearly quantitative yields.

Acknowledgment

This work was funded by the Project QLK3-CT2001-00403 "BIOMEX" as part of European Community Fifth Framework Programme and supported by the Staatssekretariat für Bildung and Forschung SBF in Switzerland.

Received for review March 1, 2008.

OP800046T