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# Reactor Operation and Scale-Up of Whole Cell Baeyer−Villiger Catalyzed Lactone Synthesis

**Steven D. Doig, Philip J. Avenell, Paul A. Bird, Patrick Gallati,† Katie S. Lander, Gary J. Lye, Roland Wohlgemuth,† and John M. Woodley***

Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, U.K.

The recombinant whole cell biocatalyst *Escherichia coli* TOP10 [pQR239], expressing cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871, was used in 1.5- and 55-L fed-batch processes to oxidize bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones, (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one. By employing a bicyclo[3.2.0]hept-2-en-6-one feed rate below that of the theoretical volumetric biocatalyst activity (275 $\mu$mol·min$^{-1}$·L$^{-1}$), the reactant concentration in the bioreactor was successfully maintained below the inhibitory concentration of 0.2−0.4 g·L$^{-1}$. In this way approximately 3.5 g·L$^{-1}$ of the combined regioisomeric lactones was produced with a yield of product on reactant of 85−90%. The key limitation to the process was shown to be product inhibition. This process was scaled up to 55 L, producing over 200 g of combined lactone product. Using a simple downstream process (centrifugation, adsorption to activated charcoal, 5-fold concentration with ethyl acetate elution, and silica gel chromatography), we have shown that the two regioisomeric lactone products could be isolated and purified at this scale.

## Introduction

Baeyer−Villiger monooxygenases (BVMOs) are a group of flavoproteins capable of the nucleophilic oxygenation of a wide range of linear or cyclic ketones yielding esters or lactones, respectively. These enzymes all use molecular oxygen and the reduced cofactor NAD(P)H as a reductant to carry out the Baeyer−Villiger type oxidation (Willetts, 1997; Kelly, 1998). The enzymatically catalyzed reaction is similar to the conventional Baeyer−Villiger oxidation, using peroxyacids, characterized over a century ago (Baeyer and Villiger, 1899), but as a result of the exquisite enantio- and regioselectivity of the enzymic catalyst, the use of BVMOs gives access to a wide range of optical pure esters and lactones (Stewart, 1998) that are not attainable using conventional chemistry.

Although over 20 different BVMOs have been identified (Willetts, 1997), the best characterized is cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 (Trudgill, 1990). CHMO is a 59-kDa FAD/NADPH-dependent monomeric enzyme. It has been used on a small scale to oxidize stereoselectively a wide range of linear and cyclic ketones, and a recent review by Stewart (Stewart, 1998) gives a comprehensive description of the catalytic versatility of this enzyme. Moreover, there are many reports demonstrating that optically pure lactone intermediates, produced via CHMO catalysis, can be subsequently used for the synthesis of a variety of valued added fine chemicals and pharmaceuticals (Alphand et al., 1990a; Alphand et al., 1990b; Alphand and Furstoss, 1992; Roberts and Willetts, 1993; Taschner et al., 1993). Therefore, there is keen interest in the development of scaleable processes for BVMO-catalyzed reactions in general.

The majority of reactions using CHMO thus far have been carried out at small scale and have used either isolated CHMO or the wild-type whole cell as the catalyst (Shipston et al., 1992; Alphand et al., 1996; Zambianchi et al., 2000). However, these two approaches suffer from severe limitations. Since the wild-type host, *A. calcoaceticus* NCIMB 9871, is a class 2 pathogen and the generation of CHMO activity requires growth on a toxic carbon source, production and use of the biocatalyst is cumbersome (Trudgill, 1990; Stewart et al., 1996). Moreover, the wild-type host also contains a lactone hydrolase, and this has been shown to decrease the yield of product on reactant (Alphand et al., 1990a). On the other hand, processes employing isolated CHMO have extra cost burdens associated with enzyme isolation. Also, since NADPH is used by the enzyme on a mole for mole basis and its cost prohibits addition of excess quantities, the implementation of an in situ cofactor recycling system is essential (Willetts et al., 1991; Kragl et al., 1996).

In an effort to overcome these generic problems, we and others (Stewart et al., 1996; Kayser et al., 1998; Chen et al., 1999; Kayser, 1999; Doig et al., 2001) have developed CHMO-expressing recombinant whole cell biocatalysts using *Escherichia coli* and *Saccharomyces cerevisiae* as hosts. Previously we have reported on the cloning and fermentation characterization of a novel strain, *E. coli* TOP10 [pQR239], which expresses CHMO at 500 to 600 U·g dwt$^{-1}$ (1 U is the amount of CHMO that catalyzes the cyclohexanone-induced oxidation of 1 $\mu$mol of NADPH per min) (Doig et al., in press). Furthermore, using this strain we have investigated the biotransformation kinetics of the oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones (−)-

* To whom correspondence should be addressed. Tel; +44(-)20-7679-3778. Fax: +44(-)20-7916-3943. Email; j.woodley@ucl.ac.uk.
† Biochemistry Department, Fluka GmbH, Industriestrasse. 25, CH-9470 Buchs, Switzerland.

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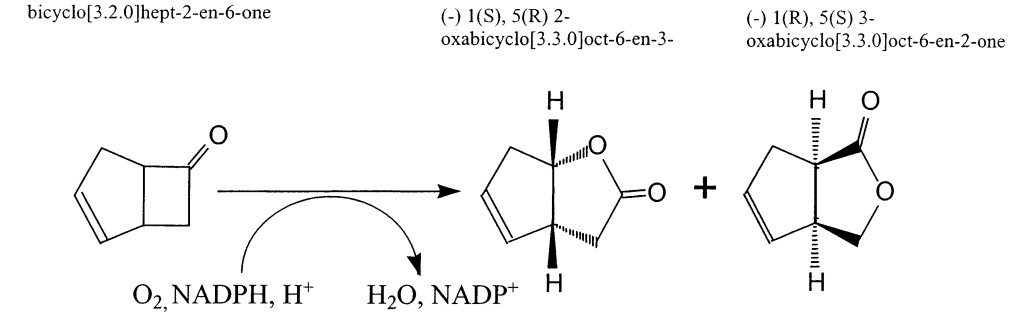


**Figure 1.** Biotransformation reaction scheme: CHMO-mediated regiodivergent oxidation of bicyclo[3.2.0]hept-2-en-6-one yielding (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one.

(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one (ee = 94%) and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one (ee = 99%) (Figure 1) (Doig et al., in press). The key kinetic characteristics of this strain were found to be severe reactant and product inhibition. The optimum specific activity of about 55 $\mu$mol·min$^{-1}$·g$^{-1}$ was observed at a ketone concentration between 0.2 and 0.4 g·L$^{-1}$, whereas at 5 g·L$^{-1}$ the activity was reduced by more than 90%. In addition, when the lactone concentration was greater than 4.5 g·L$^{-1}$ the specific activity was reduced to zero. With these characteristics in mind, we previously proposed that reactant feeding and continuous product removal should be considered to optimize the final product concentration and to maximize the yield of product on reactant for development of a scalable biocatalytic process.

The first aim of the work reported here was to operate a bench-scale (1.5 L) biotransformation process using the *E. coli* TOP10 [pQR239] whole cell biocatalyst for the oxidation of bicyclo[3.2.0]hept-2-en-6-one. Our primary objective was to optimize the final product concentration and yield of lactone on ketone to simplify the subsequent downstream product isolation. In the second stage of the work we aimed to scale up the entire fermentation, biotransformation, and product recovery process to 55 L and in this way demonstrate the technical viability of the biocatalytic Baeyer−Villiger oxidation.

## Materials and Methods

**Chemicals.** The constituents of the growth media (glycerol, yeast extract, NaCl, and tryptone) used for the *E. coli* TOP10 [pQR239] fermentation were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Bicyclo[3.2.0]-hept-2-en-6-one was kindly donated by Fluka Chemie AG (Buchs, Switzerland). Glycerol assay kits were obtained from Digen Ltd. (Wheatley, Oxford, U.K.). Ethyl acetate was obtained from Aldrich Chemical Co (Poole, Dorset, U.K.), and granular activated carbon was obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.).

**Analytical.** ***Gas Chromatography.*** Gas chromatography (GC) was used to quantify the concentrations of bicyclo[3.2.0]hept-2-en-6-one and corresponding lactones. Samples (1$\mu$L) were injected into a XL-2 gas chromatograph (Perkin-Elmer, Norwalk, CT) fitted with an AT-1701 column (30 m × 0.54 mm) (Alltech, Carnforth, Lancs, U.K.), and the concentration was determined from an external calibration curve. The GC temperature program used was 100 °C held for 5 min and then increased at 10 °C·min$^{-1}$ to 240 °C. Calibration curves for bicyclo[3.2.0]hept-2-en-6-one and (−)-(1*R*,5*S*)-2-oxabicyclo[3.3.0]oct-6-en-3-one had a coefficient of variance of ±3% (quoted at the 95% confidence interval) based on six independently prepared standard solutions (concentration range 50−2000 mg·L$^{-1}$, $R^2$ > 98%).

***Glycerol Assay.*** Samples were centrifuged prior to analysis to remove cells and debris. Glycerol concentrations were determined using a linked enzymatic based assay kit obtained from Digen Ltd. (Wheatley, Oxford, U.K.). In this method, glycerol was first phosphorylated by adenosine triphosphate using glycerokinase, yielding adenosine diphosphate. This was then used to dephosphorylate phosphoenolpyruvate catalyzed by pyruvate kinase. Finally, the resultant pyruvate was then reduced by lactate dehydrogenase and NADH to yield lactate and $NAD^+$. Since NADH absorbs strongly at a wavelength of 340 nm, the concentration of glycerol could be determined by the total difference in absorbance at this wavelength on completion of the above reactions. The error associated with this assay was ±0.07 g·L$^{-1}$ based on six independently prepared standard solutions (concentration range 4.3−9.3 mg·L$^{-1}$).

***E. coli*** **TOP10 [pQR239] Cultivation and Fermentation.** The fermentation procedure used in this work was described previously (Doig et al., in press). Whole cell biocatalysts were produced in 1.5-L fermentations using a complex growth media (10 g·L$^{-1}$ each of glycerol, NaCl, yeast extract, and tryptone peptone, and 50 mg·L$^{-1}$ ampicillin). Frozen (−70 °C) stock cultures (1 mL) were thawed and inoculated into 1-L shaken flasks (working volume, 0.1 L) and incubated at 37 °C overnight with reciprocal shaking (250 rpm). This culture was then inoculated into a LH 210 series 2-L stirred tank fermenter (working volume 1.5 L) (Bioprocess Engineering Services, Charing, Kent, U.K.) where the pH was maintained at 7 (±0.1) via the metered addition of 3 M KOH and 3 M $H_3PO_4$ and the temperature at 37 °C. The vessel was aerated with 0.67 vvm (volume per volume per minute) air via a submerged sparger. The culture was grown until the optical density (at 670 nm), measured using a Kontron Uvikon 922 variable wavelength spectrophotometer (Watford, Herts, U.K.), reached 8 and then 0.1 w/v L-arabinose was added to induce CHMO expression. The culture was grown for a further 3 h until the OD at 670 nm reached 12. Typically, 5 g dwt·L$^{-1}$ with a specific intracellular CHMO activity between 400 and 600 U·g dwt$^{-1}$, determined from cyclohexanone-induced NADPH consumption at pH 9, was produced (Doig et al., in press).

**Whole Cell Biotransformations.** ***1.5-L Scale.*** A LH 210 series 2-L stirred tank fermenter was used for all fed-batch biotransformations. pH was controlled at 7 (±0.1) via the metered addition of 3 M KOH and 3 M $H_3PO_4$, and the temperature at 37 °C. The vessel was aerated with 1 vvm air via a submerged sparger, and the impeller speed was controlled to maintain the DOT above zero. DOT was measured by a polarographic oxygen electrode (Ingold Messtechnik, Urdorf, Switzerland). Neat bicyclo[3.2.0]hept-2-en-6-one was continuously delivered

to the bioreactor from an external reservoir via a peristaltic pump (Gilson, Villiers, Le Bel, France). The ketone feed rate was varied from 1.25 to 4.8 g·h$^{-1}$. Biotransformations were carried out using either whole fermentation broth (i.e., using cells suspended in fermentation broth directly after the fermentation) or cells harvested by centrifugation (4000 rpm, Megafuge 1.0, Heraeus Sepatech, Brentwood, Essex, U.K.) and resuspended in an equal volume of 50 mmol phosphate buffer ($Na_2HPO_4$, $KH_2PO_4$). In both cases the media was supplemented with 10 g·L$^{-1}$ glycerol prior to feeding ketone, and samples (2 mL) were taken periodically, centrifuged (13000 rpm, Biofuge 13, Heraeus Sepatech, Brentwood, Essex, U.K.), and analyzed by GC.

***55-L Scale.*** Pilot-scale fed-batch biotransformation was performed in a LH series 1075 stirred tank fermenter (Bioprocess Engineering Services, Charing, Kent, U.K.) using whole fermentation broth and was carried out directly after the fermentation. The growth media (49 L) was sterilized in situ by passing steam through the vessel's heating jacket, and after the addition of 50 mg·L$^{-1}$ of filter-sterilized ampicillin (0.2 $\mu$m, Demi Nypor, Dominick Hunter, Birtley, Co. Durham, U.K.) the vessel was inoculated with 1 L of *E. coli* TOP10 [pQR239] culture grown overnight in 4 × 1-L shaken flasks (working volume, 250 mL). The pH was maintained at 7 (±0.1) via the metered addition of 3 M KOH and 3 M $H_3PO_4$, and the temperature at 37 °C. The vessel was aerated with 0.5 vvm air via a submerged sparger. The composition of the exhaust gas was determined by mass spectrometry (Prima 600, VG Gas Analysis, Winsford, Cheshire, U.K.) and data logging and exhaust gas measurements were recorded with the RT-DAS program (real-time data acquisition system) (Acquisition Systems, Guildford, Surrey, U.K.). CHMO expression was induced by the addition of 0.1% w/v of L-arabinose after the OD measured at 670 nm reached 8 and the biotransformation was started 3 h later by the addition of 10 g·L$^{-1}$ glycerol and 5 L of 10 g·L$^{-1}$ bicyclo[3.2.0]hept-2-en-6-one. At this point, continuous feeding of bicyclo[3.2.0]hept-2-en-6-one began from an external reservoir of neat ketone via a peristaltic pump at 55 g·h$^{-1}$ (1 g·L$^{-1}$·h$^{-1}$).

**Downstream Processing.** Downstream processing and product recovery were only performed at the 50-L scale

***Centrifugation.*** At the end of the fed-batch biotransformation the cells were removed from the lactone-containing broth by tubular bowl centrifugation using a Carr Powerfuge P6 (Carr Separations, Franklin, MA) (15300 rpm, 1-L bowl volume with continuous discharge) at a flow rate of 1 L·min$^{-1}$. The bowl was cooled to 4 °C with a glycol circulation system at −20 °C. The clarified broth was stored at 4 °C until required.

***Solid Adsorbent and Solvent Extraction.*** Two cylindrical glass columns (volume = 2 L each) were loosely packed with 2 kg of prewashed (RO water) activated carbon (particle size = 0.85−1.7 mm). The clarified broth was peristaltically pumped at 1 L·h$^{-1}$ to each column, in a single pass and in upflow mode. The two columns were run in parallel. The outlet ketone and lactone concentrations were measured by GC. Once all the aqueous phase had been passed through the activated carbon, ethyl acetate was recirculated through each column at 2 L·h$^{-1}$, from four 2.7-L reservoirs, until the lactone concentration in the solvent phase came to steady state.

***Purification of* (−)-*(1S,5R)*-2-*oxabicyclo*[3.0]*oct*-6-*en*-3-*one and* (−)-(1*R*,5*S*)-3-*oxabicyclo*[3.0]*oct*-6-*en*-2-*one.*** The ethyl acetate extract was vacuum-dried (Buchi Rotavapor R-3000, Flawil, Switzerland), and 35 g of the resulting residue was redissolved in 50 mL of petrol ether/*tert*-butylmethyl ether (5:1). This was then loaded onto a 2-kg silica gel 60 column and fractionated using 2 L of petrol ether/*tert*-butylmethyl ether (5:1). Pure (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one crystals were obtained by drying those fractions containing only one of the regioisomers.

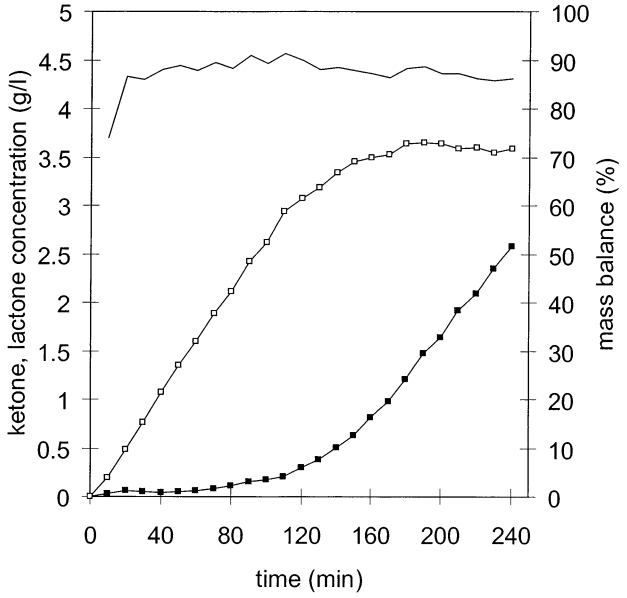


**Figure 2.** Fed-batch biotransformation (1.5 L) using whole broth showing ketone (■) and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]; 37 °C, pH 7, initial glycerol concentration = 10 g·L$^{-1}$, ketone feeding rate = 1.655 g·L$^{-1}$·h$^{-1}$.

## Results and Discussion

**Bench-Scale (1.5 L) Fed-Batch Biotransformation at Optimum Feeding Rate.** From previous kinetic characterization we have found that reactant inhibition is a key characteristic of the *E. coli* TOP10 [pQR239] whole cell catalyzed oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding lactones (Doig et al., in press). To address this issue we have used reactant feeding to maintain a low ketone concentration in an attempt to maximize the specific activity and hence final product concentration attainable. The maximum specific activity of the whole cell catalyst was about 55 $\mu$mol·min$^{-1}$·g$^{-1}$, and this occurred at a reactant concentration between 0.2 and 0.4 g·L$^{-1}$. Since the batch fermentation yielded 5 g dwt·L$^{-1}$ biocatalyst, when used under optimum conditions the maximum volumetric product formation rate could have been 275 $\mu$mol·min$^{-1}$·L$^{-1}$ (2.05 g·L$^{-1}$·h$^{-1}$).

Figure 2 shows the ketone and combined lactone ((−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one) concentration profiles during a fed-batch biotransformation carried out in whole broth (i.e., the cells were not harvested after the fermentation) at a ketone feeding rate of 255 $\mu$mol·min$^{-1}$·L$^{-1}$ (1.655 g·L$^{-1}$·h$^{-1}$). A feeding rate below the maximum was chosen to reduce the possibility of "over-feeding".

Since the biocatalyst was used in the fermentation broth and an additional supply of glycerol was made, there was possibility that it may have continued to grow

during the biotransformation. Over 240 min of continuous ketone feeding, the biocatalyst concentration increased by about 15%, from 5 to 5.75 g dwt·$L^{-1}$.

From Figure 2 it is clear that over the first 100 min of the biotransformation the product formation rate was approximately linear at about 220 $\mu$mol·$min^{-1}$·$L^{-1}$ and the ketone concentration remained at a pseudo-steady-state concentration of 0.1−0.2 g·$L^{-1}$. However, after 100 min the product formation rate decreased and as a consequence the ketone concentration began to increase. The final product concentration achieved was 3.5 g·$L^{-1}$, giving a yield of product on biocatalyst of 0.61 g·$g^{-1}$. Figure 2 also shows the percentage mass balance based on the ratio of combined mass of ketone and lactone present in the reactor at a given time to the total mass of ketone added to the system at that time. During this run nearly 90% of the ketone added to the system could be accounted for, although only 46% of it was converted to lactone.

The most likely explanation for these observations was that the product accumulating in the reactor inhibited the biotransformation. Previously, we evaluated the effect of the combined (i.e., an equimolar mixture of the two regioisomers) lactone concentration on the specific activity of the biocatalyst and showed that the exogenous addition of 4.5 g·$L^{-1}$ reduced the activity to zero (Doig et al., in press). However, we also demonstrated that the specific activity was limited by mass transfer of the reactant and/or product across the cell membrane. Therefore, it seems likely that the effect of exogenous lactone addition may appear less inhibitory than the similar effect observed with de novo synthesis. Hence, it is probable that product inhibition was the cause of cessation of the biotransformation at a product concentration of 3.5 g·$L^{-1}$ and the implementation of a continuous product removal technique (in situ product removal, ISPR (Lye and Woodley, 1999)) would seem appropriate. As a result of the hydrophilic nature of the lactone products, implementation of ISPR is difficult, and with the current state of the art the final product concentration achievable in this system is limited to about 3.5 g·$L^{-1}$.

Figure 3 shows the results from a similar fed-batch experiment (feeding rate = 1.748 g·$L^{-1}$·$h^{-1}$, 270 $\mu$mol·$min^{-1}$·$L^{-1}$). However, to increase the final yield of product on reactant the ketone feed stream was shut off after 120 min. The product evolution profile during this run was similar to Figure 2, and by cessation of the ketone feed the final yield of lactone on ketone was increased to 86% and the residual ketone concentration was not detectable. This has clear benefits for downstream processing. Furthermore, the final product concentration achieved was again 3.5 g·$L^{-1}$, supporting the hypothesis that product inhibition was limiting the reaction.

Figure 3 also shows the glycerol concentration profile during the process. The consumption rate was approximately 1.75 g·$L^{-1}$·$h^{-1}$ (19 mmol·$L^{-1}$·$h^{-1}$), and it was clear that the initial addition of 10 g·$L^{-1}$ was sufficient for completion of the biotransformation. Furthermore, the yield of lactone on glycerol during the linear product formation region (0−100 min) was about 0.75 mol·$mol^{-1}$. This glycerol was used by the cell as a reductant to supply the required NADPH for the biotransformation, and this issue is discussed in more detail later.

**Bench-Scale (1.5 L) Fed-Batch Biotransformation at Suboptimal Feeding Rates.** Figures 4 and 5 show the ketone and combined lactone concentration profiles at feeding rates of 0.843 and 3.154 g·$L^{-1}$·$h^{-1}$ (131 and 487 $\mu$mol·$min^{-1}$·$L^{-1}$), respectively. At the lower feeding rate (Figure 4), as expected, the rate of product formation

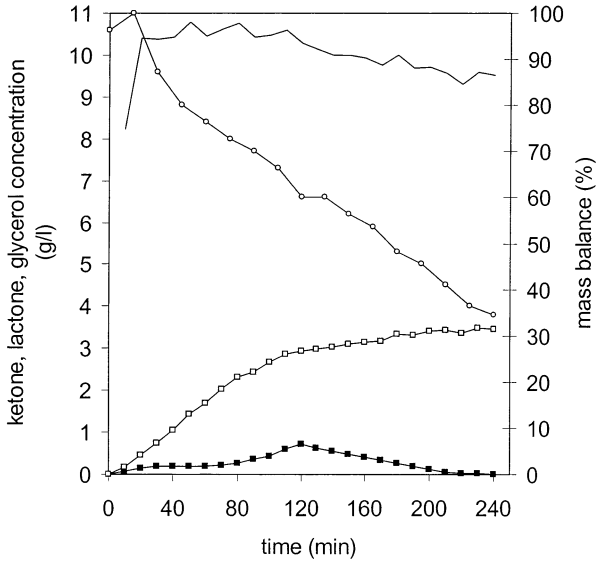


**Figure 3.** Fed-batch biotransformation (1.5 L) using whole broth showing glycerol (○), ketone (■), and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]. The ketone feed was shut off after 120 min; 37 °C, pH 7, initial glycerol concentration = 10 g·$L^{-1}$, ketone feeding rate = 1.748 g·$L^{-1}$·$h^{-1}$.

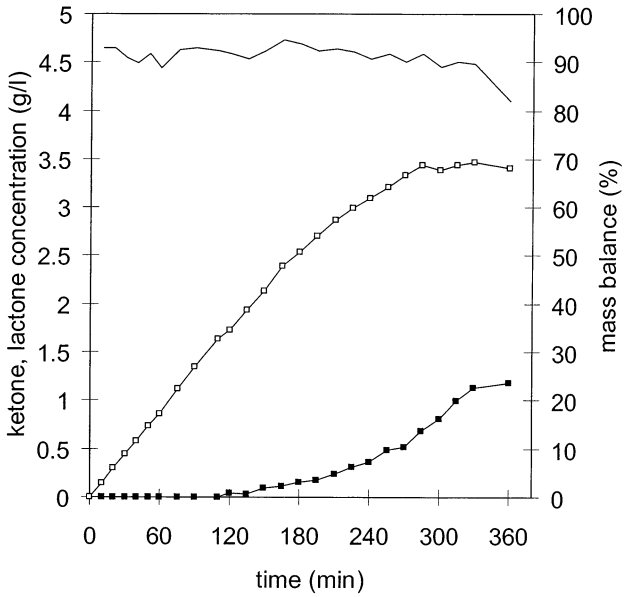


**Figure 4.** Fed-batch biotransformation (1.5 L) using whole broth showing ketone (■) and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]; 37 °C, pH 7, initial glycerol concentration = 10 g·$L^{-1}$, ketone feeding rate = 0.843 g·$L^{-1}$·$h^{-1}$.

was proportionately slower and the time taken to reach the final product concentration of 3.5 g·$L^{-1}$ was approximately twice as long. This information provides evidence that the limited final product concentration observed in Figure 2 was not due to the instability of the biocatalyst and in this way provides still further evidence that product inhibition was the key limitation.

At the higher feeding rate (Figure 5) it is clear that the ketone concentration increased dramatically from the start of the biotransformation. This occurred because the rate of feeding was higher than the maximum volumetric biocatalyst activity. Furthermore, because the ketone concentration rapidly increased, the biocatalyst activity

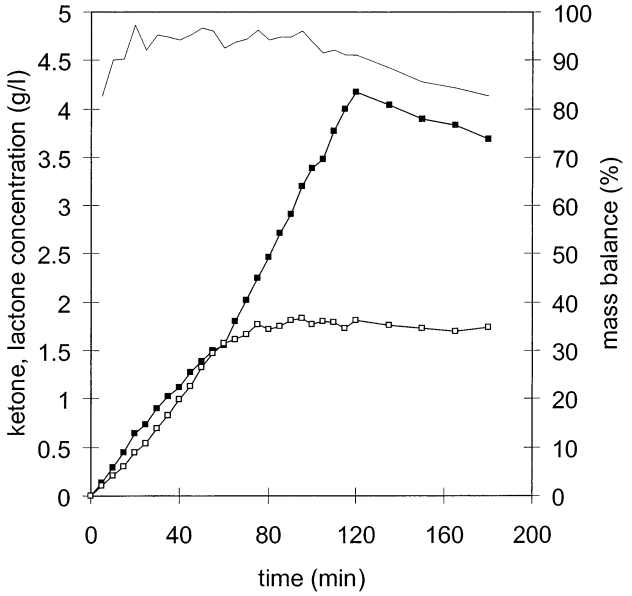


**Figure 5.** Fed-batch biotransformation (1.5 L) using whole broth showing ketone (■) and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]. Ketone feed was shut off after 130 min; 37 °C, pH 7, initial glycerol concentration = 10 g·L$^{-1}$, ketone feeding rate = 3.154 g·L$^{-1}$·h$^{-1}$.

significantly decreased during the process. This effect was due to reactant inhibition (Doig et al., in press) and resulted in a final product concentration of less than 2 g·L$^{-1}$. Therefore, it is clear that control of the ketone feed rate is necessary in order to maintain it below the maximum volumetric biocatalyst activity. However, if the feeding rate is lower than the optimum then it does not adversely affect the final product yield.

**Bench-Scale (1.5 L) Fed-Batch Biotransformations Using Resuspended Cells.** It may be desirable to use the biocatalyst after harvesting from the fermentation broth, either to simplify downstream processing or to concentrate the biocatalyst and hence to separate the fermentation and biotransformation (Lilly et al., 1994). Figure 6 shows the ketone, lactone, and glycerol concentration profiles from a fed-batch biotransformation carried out using cells resuspended in 50 mmol phosphate buffer ($Na_2HPO_4$, $KH_2PO_4$, pH 7) at a ketone feed rate of 1.87 g·L$^{-1}$·h$^{-1}$. The biocatalyst was harvested using a lab-scale centrifuge and was resuspended by adding the wet cell paste to the stirred tank reactor operating at an agitator speed of 800 rpm ($Re \approx 33{,}000$). It is clear that the final combined lactone concentration was below 2.3 g·L$^{-1}$, 30% lower than that routinely achieved using the whole broth, at 3.5 g·L$^{-1}$. However, the rate of glycerol consumption was approximately the same, being 1.8 g·L$^{-1}$·h$^{-1}$, and the overall mass balance of ketone and combined lactone was about 85%. Therefore, using the resuspended biocatalyst resulted in a lower final product concentration and thus lower yield of product on biocatalyst (0.45 g·g$^{-1}$). We speculate that this could have been due to one of two factors. Either the action of harvesting and resuspending damaged the biocatalyst and thus reduced its active lifetime, or the spent fermentation broth contained "nutrients" that allowed the biocatalyst lifetime to be extended. In an effort to resolve this issue we resuspended cells in their spent broth. Figure 7 shows similar profiles observed using cells harvested and directly resuspended in their spent fermentation broth. It is clear that the product evolution profile and final

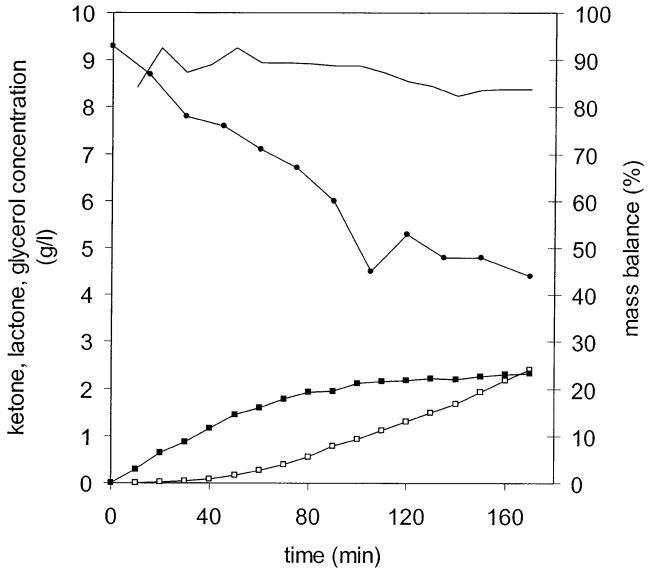


**Figure 6.** Fed-batch biotransformation (1.5 L) using biocatalysts resuspended in 50 mmol·L$^{-1}$ phosphate buffer showing glycerol (○), ketone (■), and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]; 37 °C, pH 7, initial glycerol concentration = 10 g·L$^{-1}$, ketone feeding rate = 1.870 g·L$^{-1}$·h$^{-1}$.

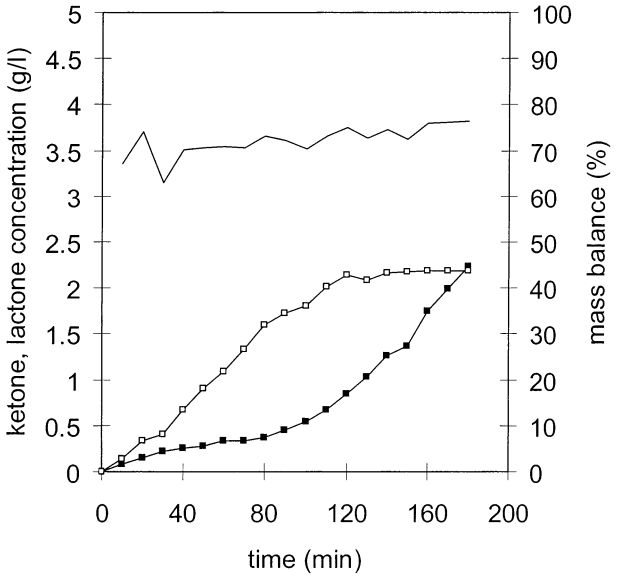


**Figure 7.** Fed-batch biotransformation (1.5 L) using biocatalysts resuspended in "spent" broth showing ketone (■) and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]; 37 °C, pH 7, initial glycerol concentration = 10 g·L$^{-1}$, ketone feeding rate = 1.807 g·L$^{-1}$·h$^{-1}$.

concentration were similar to those in Figure 6, and therefore it seems that the action of harvesting and resuspending caused the suboptimal productivity. Although the precise mechanism causing this effect was not determined, it is apparent that the biotransformation should be carried out directly after the growth stage using whole broth. However, harvesting and resuspension could be considered if the benefits mentioned above outweighed the 30% reduction in product yield.

**Pilot-Scale Biotransformation.** The fed-batch biotransformation was scaled up to 55 L, using the insights gained from the 1.5-L scale, with the objective of demonstrating the pilot-scale production of multi-100 g

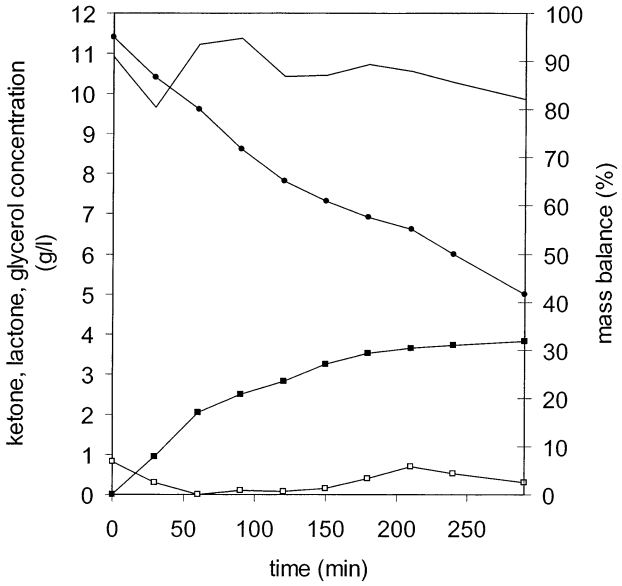


**Figure 8.** Pilot-scale fed-batch biotransformation using whole broth showing glycerol (○), ketone (■), and lactone (□) concentration profiles and the % mass balance (—) [defined as (mass of ketone + mass of lactone present in the reactor)/(total mass of ketone added to the reactor)]; 37 °C, pH 7, initial glycerol concentration = 10 g·L$^{-1}$, ketone feeding rate = 1 g·L$^{-1}$·h$^{-1}$.

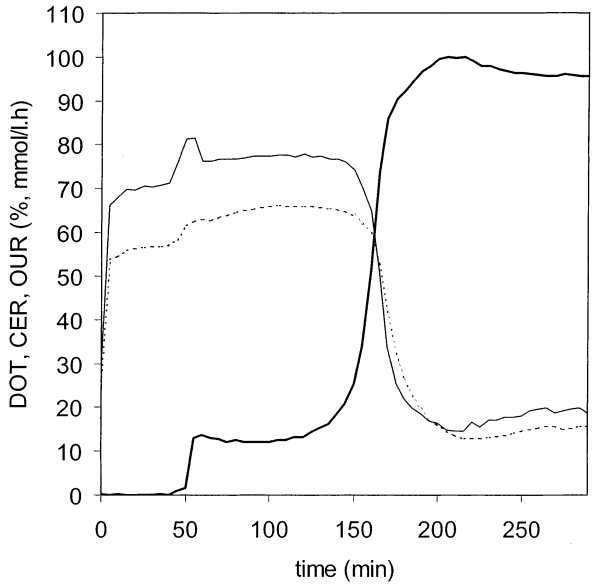


**Figure 9.** Pilot-scale fed-batch biotransformation: online DOT (—), OUR (–), and CER (···).

quantities of the lactones. We also intended to explore the extraction and purification of the two regioisomeric lactones to verify the technical feasibility of the biocatalytic production of Baeyer–Villiger products. It was not our objective to thoroughly examine the downstream processing of the biotransformation.

Results from the batch fermentation are not shown, although the profiles were similar to those we have reported previously at the 1.5- and 300-L scales (Doig et al., 2001) yielding 5 g dwt·L$^{-1}$ biocatalyst with an intracellular CHMO titer of 400 U·g$^{-1}$ (based on the cyclohexanone induced oxidation of NADPH). Two modifications were made to the fed-batch biotransformation at the 50-L scale. First, the ketone feeding rate was set at 1 g·L$^{-1}$·h$^{-1}$ (beneath the optimum of about 1.8 g·L$^{-1}$·h$^{-1}$). This was implemented since the dissolution rate of ketone at the pilot scale was unknown and it minimized the possibility of "over-feeding" the system. Previously we showed that this lower feeding rate should not result in a reduction in the final product concentration and therefore should be satisfactory. Second, an initial batch addition of 1 g·L$^{-1}$ equivalent of ketone (as an aqueous solution) was made so that sufficient ketone was added to the system in the first hour for near maximum specific activity.

Figures 8 and 9 show the concentration–time profiles and online and exit gas analysis profiles from the pilot-scale biotransformation. Ketone feeding was terminated after 210 min, and the final reactant and product concentrations were 0.29 and 3.82 g·L$^{-1}$, respectively. The overall mass balance of reactant and product was 82%, and the yield of lactone on ketone was 76%. In total, 210 g of combined lactone was produced. The rate of glycerol consumption was slightly lower than that observed at the 1.5-L scale (1.33 g·L$^{-1}$·h$^{-1}$), which was expected as a result of the lower volumetric biotransformation rate. Unfortunately, from Figure 9, the DOT during the first 40 min of the process was near to zero, and therefore the impeller speed was manually increased, resulting in an increase in the DOT to about 10–15%. However, the specific lactone production rate during this initial period was 55 $\mu$mol·min$^{-1}$·g$^{-1}$, and therefore it seems that oxygen mass transfer was not limiting. After 140 min the DOT dramatically increased to 100% saturation and this coincided with a significant reduction in the product formation rate. The average lactone formation rate after this 140-min period was 11 mmol·L$^{-1}$·h$^{-1}$.

During the first 140 min the OUR was at a pseudo-steady state between 70 and 75 mmol·L$^{-1}$·h$^{-1}$, and as expected this decreased rapidly to about 10 mmol·L$^{-1}$·h$^{-1}$ once lactone production ceased. The CER during this period was about 60 mmol·L$^{-1}$·h$^{-1}$. Moreover, it should be possible to predict these pseudo-steady-state OUR and CER values from the measured glycerol consumption rate. If we assume the glycerol consumed by the biocatalyst was completely oxidized (which was likely since oxygen was in excess) then per mol of glycerol oxidized, 3 mol of $CO_2$ would have been produced and 3.5 mol of $O_2$ would have been consumed. The measured glycerol consumption rate during this run was 14.4 mmol·L$^{-1}$·h$^{-1}$, and therefore the predicted OUR based solely on glycerol consumption was 50.4 mmol·L$^{-1}$·h$^{-1}$. However, the total OUR was a combination of that for glycerol and ketone oxidation. Therefore, since 1 mol of oxygen was needed per mol of lactone formed, the predicted overall OUR was 50.4 + 11 = 61.4 mmol·L$^{-1}$·h$^{-1}$. The stoichiometrically predicted CER was 43 mmol·L$^{-1}$·h$^{-1}$. Therefore, it seems that is there a reasonable mass balance not only of ketone and lactone but also of glycerol and oxygen. Furthermore, using the experimental data, an overall molar biotransformation stoichiometry, at the pilot scale, can be described:

$$1.32 \text{ mol ketone} + 1.37 \text{ mol glycerol} + 6.91 \text{ mol } O_2 \rightarrow 1 \text{ mol lactone} + 5.71 \text{ mol } CO_2$$

**Pilot-Scale Product Recovery.** Fifty-five liters of biotransformation broth, containing 3.82 g·L$^{-1}$ combined lactone and 0.3 g·L$^{-1}$ ketone, was processed first by centrifugation, followed by solid adsorption, solvent elution, and silica gel chromatography, as described in Materials and Methods and schematically in Figure 10.

Over 95% of the biocatalyst was removed from the broth using the Carr Powerfuge P6 operated at 1 L·min$^{-1}$, and 100% of the solubilized lactone remained in the clarified liquor. This liquor was then passed through

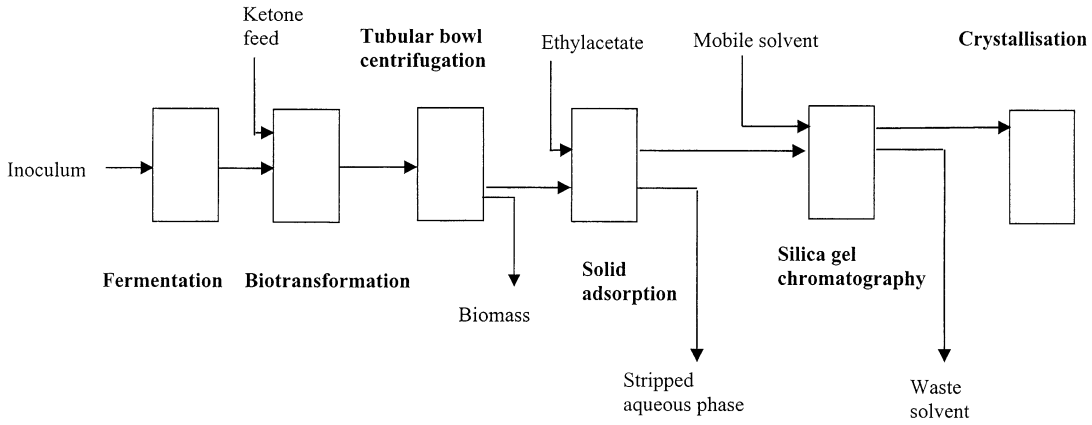


**Figure 10.** Schematic presentation of the pilot-scale process flowsheet.

activated carbon at 2 L·h$^{-1}$. The stripped aqueous phase passing out of the packed columns was completely depleted in residual ketone, while 92% of the combined lactone was removed. Once the solid adsorbent had been thoroughly drained, 11 L of ethyl acetate was used to elute the lactones from the activated carbon. The combined lactone concentration came to steady state in the eluant after 10 h, and approximately 90% of the adsorbed lactone was recovered. Five hundred milliliters of the elution solvent was lost as a result of evaporation. It should be stressed that these operations were not optimized, and a more thorough examination of these primary downstream processing steps will be published elsewhere. Nevertheless, 83% of the lactone produced was extracted from the biotransformation media and concentrated 5-fold (via elution in one-fifth the original volume), resulting in 10.5 L of ethyl acetate with a lactone concentration of 16.5 g·L$^{-1}$.

In the final steps of product purification the two regioisomeric lactones were separated by silica gel chromatography and crystallized. The ethyl acetate extract was vacuum-dried, and the resultant residue contained 38% (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one and 31% (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one. The remaining material was a combination of unreacted ketone and some unidentified compounds. Thirty-five grams of this dried extract was redissolved in 50 mL of a 5:1 mixture of petroleum ether and *tert*-butylmethyl ether and loaded onto the silica column. Following elution with 2 L of the same solvent mixture, 33 fractions were collected. Fractions 1−17 contained the various unidentified impurities and ketone. Fractions 18−20 contained (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one, fractions 21 and 22 contained a mixture of the two lactones, and fractions 23−33 contained pure (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one. The pure lactone fractions (18−20 and 23−33) were dried to yield 5.3 g of pure (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one and 8.3 g of pure (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one.

## Conclusions

The *E. coli* TOP10 [pQR239]-catalyzed Baeyer−Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones was carried out at 1.5- and 55-L scales using a reactant feeding strategy to overcome inhibition. By continuously feeding the reactant to the biocatalyst at a rate below the theoretical maximum volumetric activity it could be maintained at subinhibitory concentrations. In this way, a final product concentration of about 3.5 g·L$^{-1}$ could be achieved with a yield on reactant of 85%. This process was successfully scaled up to pilot-plant scale, producing 210 g of combined lactone in one batch, of which 174 g was extracted. Finally, it was also shown that a fraction (24 g) of these extracted lactones could be separated and purified by silica gel chromatography to yield pure (−)-(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. Ensuring there was no ketone in the reactor exit stream is clearly important to the downstream processing. Further improvements can also be made by substituting the silica gel chromatography with distillation, thus reducing the solvent inventory.

Overall, the key limitation to productivity in this process was product inhibition, resulting in a low final product concentration and poor yield on biocatalyst. Furthermore, because of the similarity of the product and the reactant and the hydrophilic nature of the combined lactones, we were not able to selectively remove the product continuously. Subsequent work will address this problem.

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