Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron 60 (2004) 781–788
Tetrahedron

An alternative bioreactor concept for application of an isolated oxidoreductase for asymmetric ketone reduction

Vicky J. Shorrock,[a] Michel Chartrain[b] and John M. Woodley[a,*]

[a]*Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK*
[b]*Bioprocess R and D, Merck Research Laboratories, P.O. Box 200, Rahway, NJ 07065, USA*

Received 19 August 2003; revised 13 November 2003; accepted 19 November 2003

Abstract—In this paper an isolated NADH dependent ketone reductase has been used to synthesise (*S*)-6-bromo-β-tetralol from 6-bromo-β-tetralone, together with commercially available formate dehydrogenase (FDH) as a recycle enzyme to produce preparative quantities of the product. Furthermore, initial experiments indicate potential for an alternative bioreactor concept via the use of a resin (XAD L-323) to bind the product (and residual substrate) of the conversion rather than the cofactors or enzymes, thus allowing a new method of recycle, potentially overcoming existing problems.

1. Introduction

Regio- and stereoselective oxidation and reduction are of paramount importance in synthetic strategies and a range of biocatalysts are available to assist.[1,2] Oxidoreductases are amongst the most useful of all the classes of enzyme to assist organic chemists.[3,4] However, there are practical problems which still prevent widespread implementation. A good example which illustrates some of these limitations is the application of a ketone reductase recently found and isolated from *Trichosporon capitatum*.[5,6] The enzyme can be used in whole cell format to effect the reduction of 6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol (a potential pharmaceutical precursor[7]) with 98% ee (Fig. 1). While the NADH cofactor, which is required, can be recycled in the cell via judicious choice of cosubstrate, other problems are still evident. The reactant is highly insoluble in aqueous environments (0.5 g/L in water) and the rate of dissolution into the reaction mix is too slow to use it as a solid. A solvent is, therefore, required to solubilise the reactant for the reaction. In addition, although the cells displayed high activity, the reactant and product adhered to the cell. Recovery of the product (6-bromo-β-tetralol) was achieved only by the addition of ethanol, which subsequently caused irreversible cellular damage and prevented the cells from being recycled. The limiting factor in the whole cell system was the reactant and product adherence to the cell. However, the solvent, which was used to dissolve 6-bromo-β-tetralone also reduced cell viability. The cells achieved a maximum productivity of 1.6 g of product per gram of cell mass, with a conversion of 63%.

As a result of these initial findings, we have more recently been investigating the isolation of this enzyme with a view to running an isolated immobilised enzyme process with a second auxiliary enzyme, FDH,[8] for NADH recycle. Formate dehydrogenase can be produced in bulk quantities from *Candida boidinii*,[9] and to date, only the use of FDH from *C. boidinii* for the regeneration of NADH has been demonstrated on a technical scale.[4] The NADH dependent

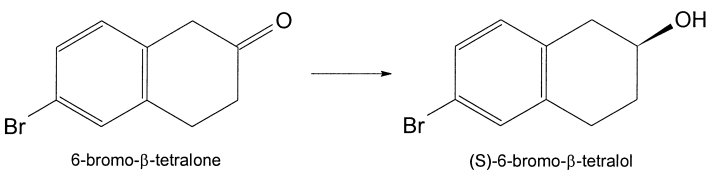

Figure 1. Conversion of 6-bromo-β-tetralone to (*S*)-6-bromo-β-tetralol by ketone reduction in *Trichosporon capitatum* (MY 1890).

Keywords: Oxidoreductase; 6-Bromo-β-tetralone; *Trichosporon capitatum*.
* Corresponding author. Tel.: +44-20-7679-3778; fax: +44-20-7916-3943; e-mail address: j.woodley@ucl.ac.uk

0040–4020/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/j.tet.2003.11.060

tetralone reductase was successfully isolated from the yeast cells of *T. capitatum* (MY 1890).[6] The enzyme exhibited a half-life of 7 h at 22 °C after the Q-Sepharose chromatography column. The half-life was increased to 90 h at 22 °C by removing the EDTA, which was a component of the protease inhibitor. The enzyme could be further purified via chromatography using hydroxy apatite and toyopearl resins sequentially.[6] However, the isolated ketone reductase did not bind effectively to the immobilisation support, while the substrate did, and this therefore, led to a re-evaluation of the logic for immobilisation.

Figure 2 indicates three schemes which can be used downstream of an isolated enzyme reactor for separation of the components. Figure 2, Scheme I employs immobilised biocatalysts(s) (B) which can be easily separated by filtration followed by a subsequent separation of cofactor (C) and product (P). This second step is not straightforward and the use of NADH attached to PEG for example, has been shown to be effective in aiding filtration.[10] In fact with such a system, both biocatalyst and cofactor can be isolated with filtration and retained for recycle to the reactor (Fig. 2, Scheme II). Clearly attaching PEG to the NADH has consequences, as does immobilisation resulting in reduced activity unless very small beads can be used to overcome diffusional limitations (and these are then difficult to filter). An alternative approach is to retain the product on a support and allow the biocatalyst(s) and cofactor to leave the reactor

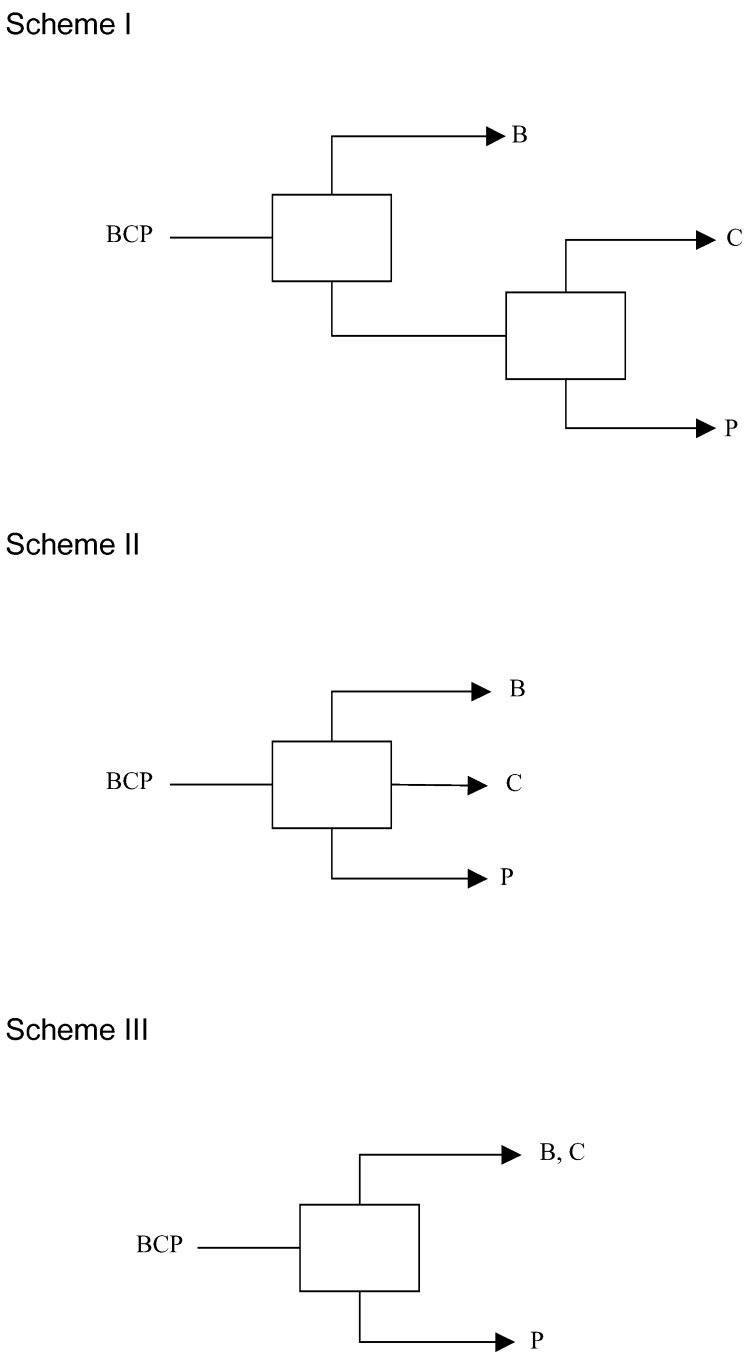

Figure 2. Possible schemes for separation of biocatalyst (B), cofactor (C) and product (P) from an isolated oxidoreductive conversion.

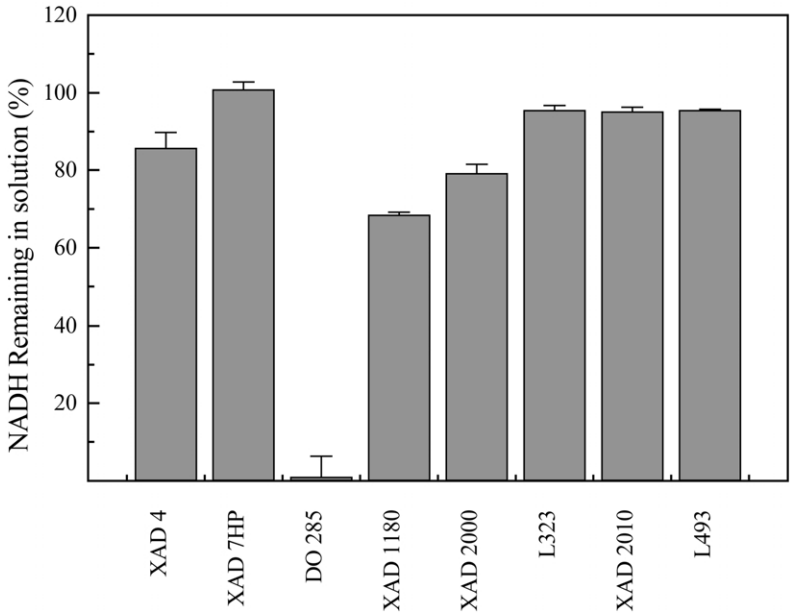

Figure 3. Resin screen for NADH binding. Error bars were calculated as standard deviations of multiple reactions.

ready for recycle. Subsequently product can be eluted. In many cases finding a product-selective carrier is particularly difficult[11,12] but a non-selective carrier will still allow effective separation for downstream processing providing a high conversion is achieved in the reactor (Fig. 2, Scheme III). In this paper, we explore such an approach using the reduction of (*S*)-6-bromo-β-tetralone as an example.

2. Results and discussion

2.1. Immobilisation of tetralone reductase

Immobilisation of tetralone reductase was ineffective as the reactant bound to the Eupergit C® beads and therefore the activity of the immobilised enzyme could not be measured. This binding property observed with Eupergit C was subsequently used as the basis for the alternative reactor concept.

2.2. Resin screen for NAD(H) binding

A range of cheap XAD resins were obtained and screened for their binding of NAD(H). If the cofactor, NADH was to be reused, then it must not adsorb on the resins (Fig. 2). Results for these trials are shown in Figure 3. Dowex optipore (DO 285) showed significant adsorbance of NADH. For effective product recovery, the resins needed to adsorb the reactant and product, not the enzymes or cofactor. The resins, which were found to be the most successful at retaining NADH in solution were XAD 7HP, XAD L-323, XAD 2010 and XAD L-493.

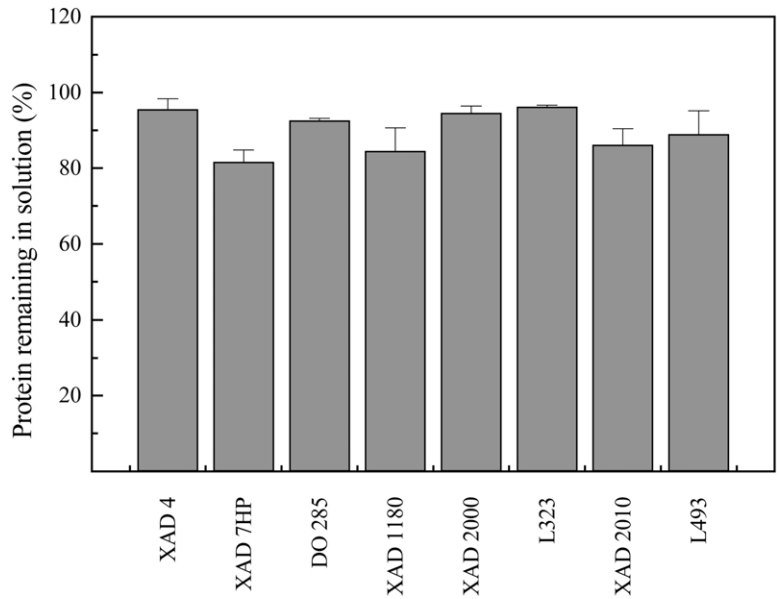

Figure 4. Resin screen for protein binding. Error bars were calculated as standard deviations of the multiple reactions.

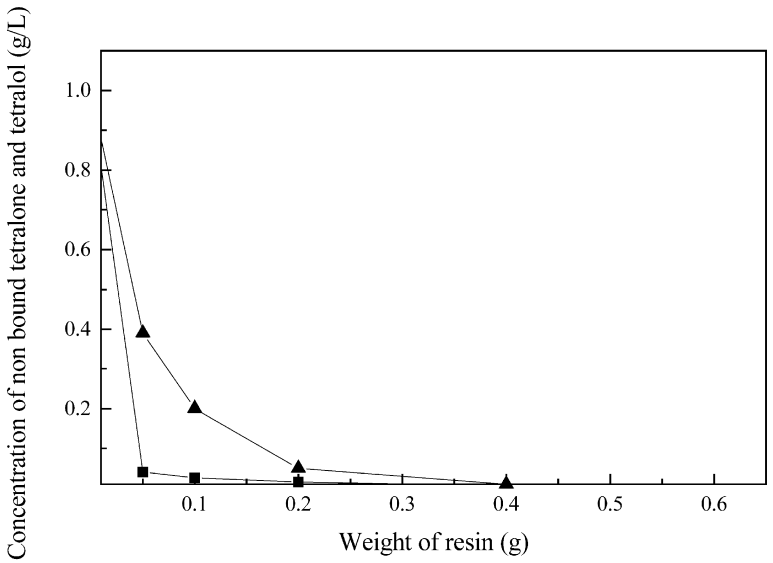

Figure 5. Adsorbance of 6-bromo-β-tetralone (■) and 6-bromo-β-tetralol (▲) on XAD L-323.

2.3. Resin screen for protein binding

The resins were also screened for their ability to adsorb the enzymes (ketone reductase and FDH) in the samples (Fig. 4). If the enzymes were to be reused, then they should not adsorb onto the resin. Little adsorbance of the enzymes was observed on the resins tested.

2.4. Binding of reactant and product on XAD L-323

If product removal was to be employed in a process, the resin used to adsorb the product and unconverted reactant must have a high affinity towards these compounds, but must reject the cofactors and the enzymes. 6-Bromo-β-tetralone was easily adsorbed onto the XAD resin L-323 (Fig. 5). An unoptimised mass of 50 mg of the resin adsorbed the 10 mg of tetralone in the solution. 6-Bromo-β-tetralol was less easily adsorbed onto the XAD resin (Fig. 5). An unoptimised mass of 200 mg of the resin adsorbed the 10 mg of 6-bromo-β-tetralol in the solution.

2.5. Bioconversion

A bioconversion was initiated using highly concentrated 6-bromo-β-tetralone dissolved in methoxyethanol, which had previously been found to be the most effective solvent.[6] This would reveal whether an efficient bioconversion could be achieved using low volumes of solvent, but high reactant concentrations. The regenerating enzyme and cosubstrate were both added to the reaction mixture in excess. Results revealed that the recycling enzyme system worked effectively. Using the initial reactant concentration of 1 g/L gave a high initial activity for the enzyme (Fig. 6) and a high conversion (Fig. 7). This initial reactant concentration of 1 g/L was used in subsequent bioreactor experiments.

2.6. Recycle bioreactor (10 mL)

A 10 mL batch bioreactor was run to prove that the bioconversion could be implemented, with subsequent cofactor and enzyme recycling, and regeneration. The

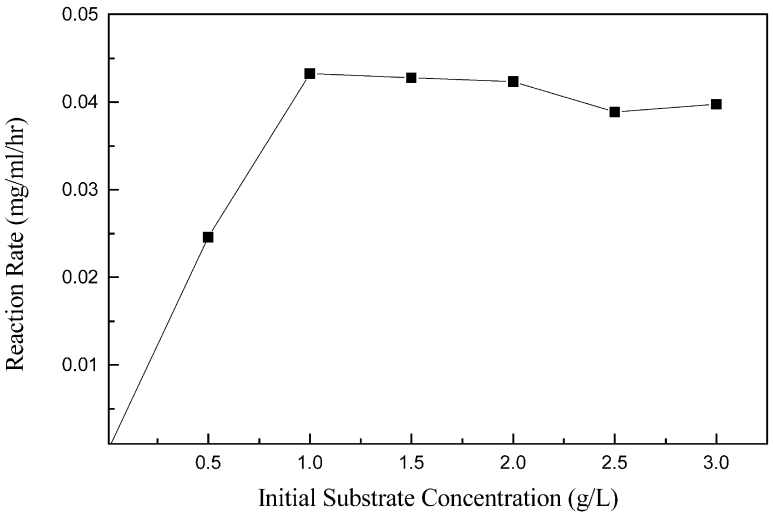

Figure 6. Effect of initial substrate concentration on the rate of reaction. 100 mL of methoxyethanol was used to dissolve 6-bromo-β-tetralone in 10 mL of reaction mixture.

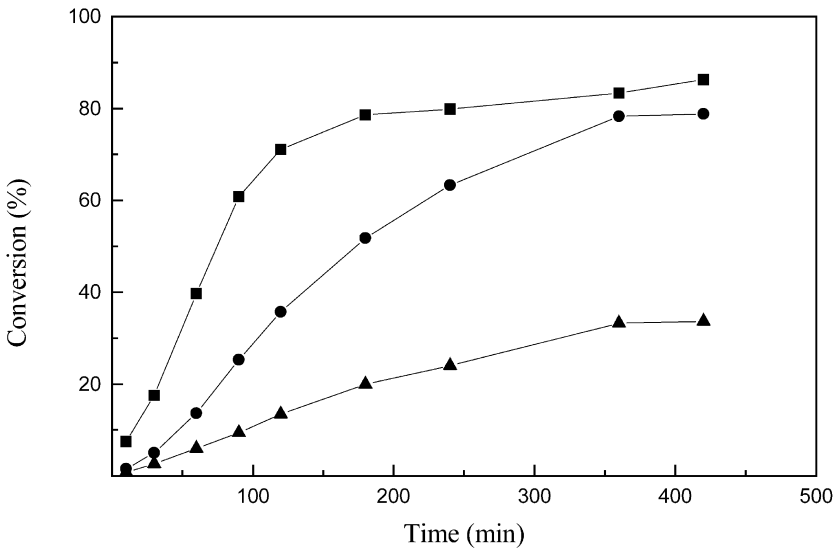

Figure 7. Bioconversion profile of 6-bromo-β-tetralone to 6-bromo-β-tetralol using methoxyethanol as the solvent with an initial reactant concentration of 1 g/L (■), 5 g/L (●), and 10 g/L (▲).

method used a batch reactor, in which all the reaction components were added simultaneously. Prior to filtration, the resin was added to the reaction components and left to adsorb the residual reactant and product, as shown in the method diagrammatically represented in Figure 8. The conversion-time profile is shown in Figure 9. A second pass was run with the enzyme and cofactor from the first batch and the reaction followed a similar reaction profile (Fig. 9),

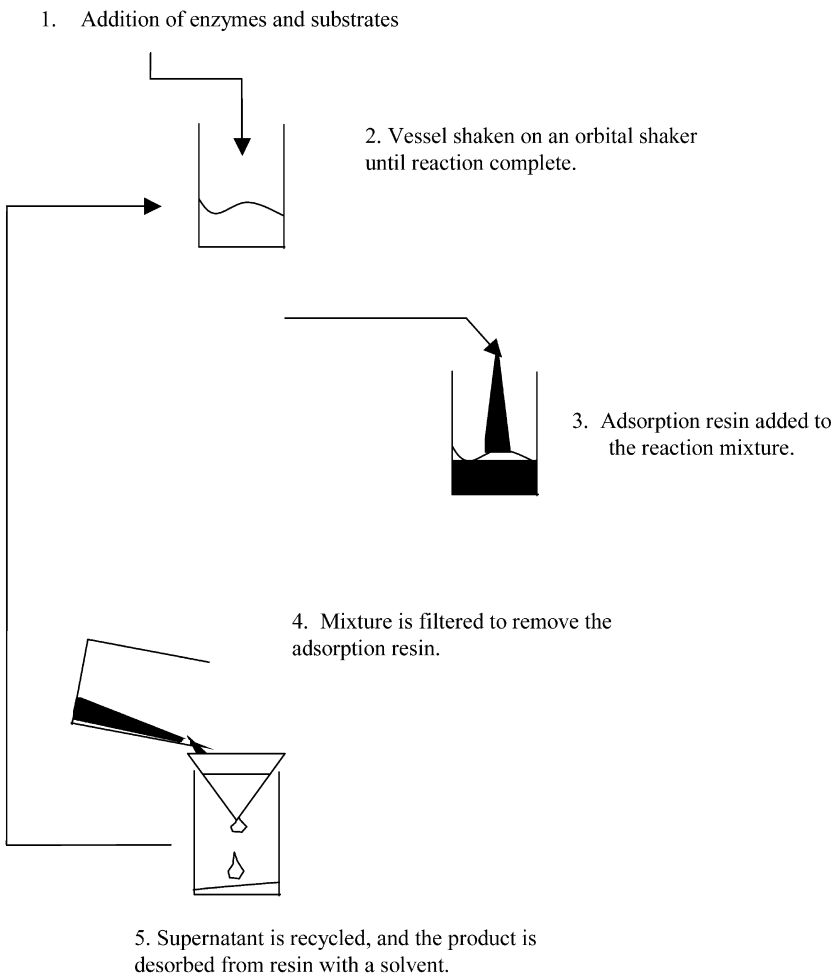

Figure 8. Reaction methodology for the batch production of 6-bromo-β-tetralol.

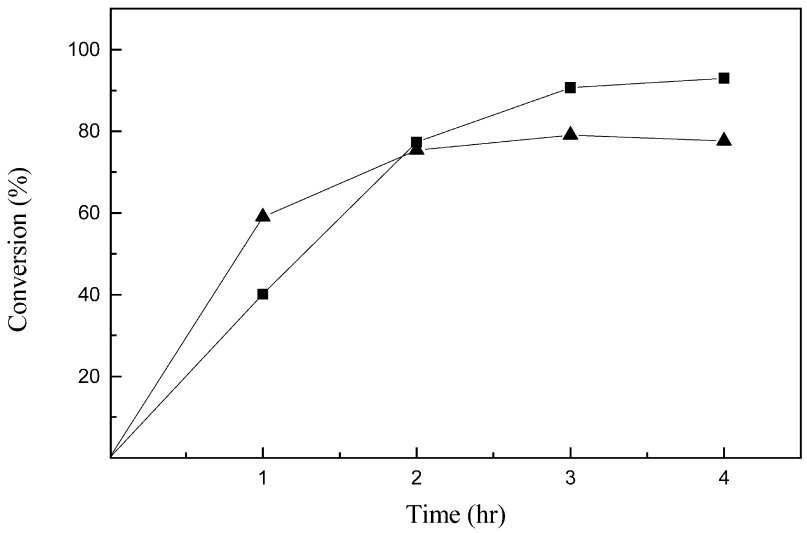

Figure 9. Bioconversion profile for a first (■) and second batch (▲), where the product and remaining reactant have been separated from the enzymes and cofactors, which have already completed a first batch reaction.

although top up enzyme was added. Complete conversion was not met in either case, with the second pass showing lower conversion than the first pass.

3. Conclusions and outlook

In this paper a new concept for operation of an isolated oxidoreductase has been outlined using immobilisation of (reactant and) product rather than enzyme(s) and cofactor. The concept has been briefly illustrated using the reduction of a ketone with an isolated reductase from yeast. The system is unoptimised but has potential for operation providing the isolated enzyme is sufficiently stable. Three options exist for implementation. First, the potential configuration of the bioreactor could be a (continuous) stirred tank reactor (Fig. 10). This would be particularly attractive since the enzyme is not immobilised onto a resin,

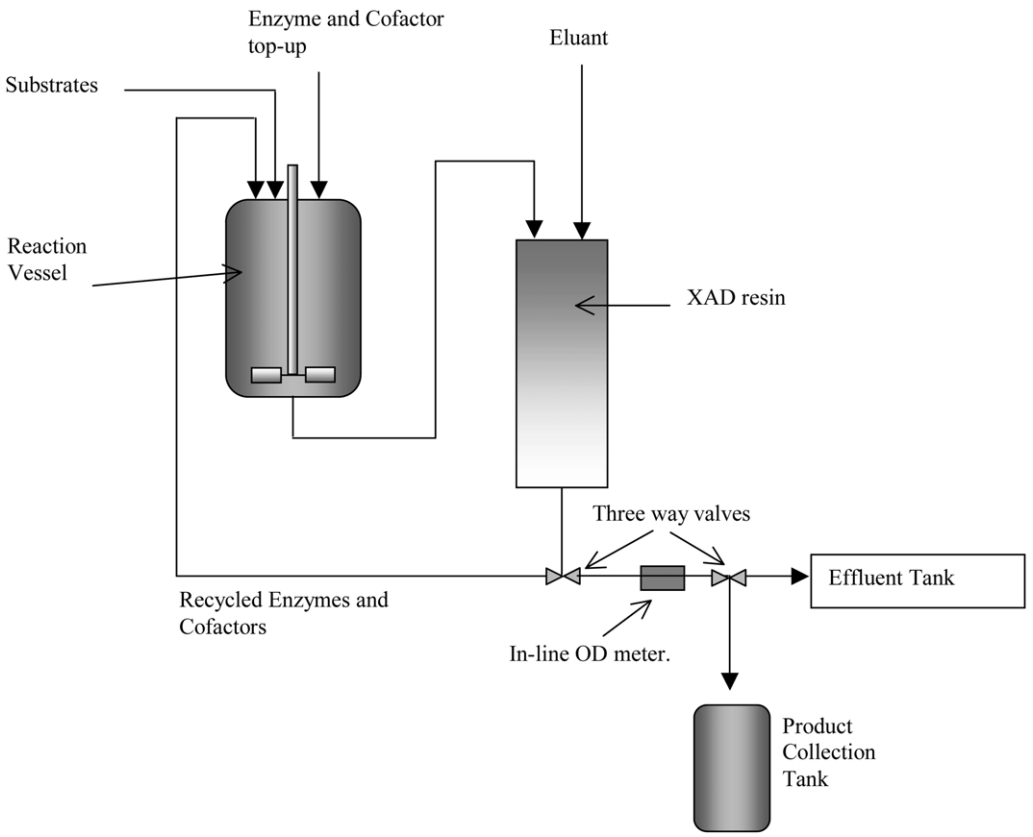

Figure 10. Possible design of the bioreactor with product removal and cofactor recycle.

and need not be retained behind a membrane. The reactants could be added to the bioreactor, whereby the enzymes and cofactors carry out the asymmetric synthesis. Since, the concentration of reactant in the reactor is the same as that in the leaving stream, toxic reactants could be handled effectively.[13] After the reaction is complete, the reaction mixture could then be passed through a column of XAD-323 resin where the product (and residual reactant) would be retained and the enzyme(s) and cofactors recycled back to the reactor where more reactant would be added. More enzyme and cofactor could be added with the reactant to top up the volume, if there are losses in the XAD column. Separately, the product would be eluted from the XAD resin using a solvent, in this case, ethanol. Based on initial experiments, using ethanol as an eluant would give a final product concentration of approximately 10 g/L. A higher product concentration could be achieved by using a solvent with a higher solubility of 6-bromo-β-tetralol, and hence a smaller volume.

Secondly, if the enzyme could be stabilised using a resin which 6-bromo-β-tetralone and 6-bromo-β-tetralol did not adhere to, the same bioreactor concept could still be used. The immobilised enzyme(s) could be retained in a first column (instead of an STR), and the cofactor and other reaction components could leave the reactor to the XAD resin on a second column, as before, and the cofactors recycled back into the bioreactor in continuous operation. This might give added stability to the enzyme(s), where operation with the isolated enzyme was not possible.

Finally, the third option could be to use an adsorbent resin as a method of substrate delivery and removal. This has previously been described for whole cell yeast mediated stereoselective ketone reductions.[14–16] In one example,[15] at scale, 3,4-methylene-dioxyphenyl acetone was reduced to the corresponding (*S*)-3,4-methylene-dioxyphenyl isopropanol in greater than 95% isolated yield and 99.9% enantiomeric excess. The supply of the reactant and the removal of the product, via a polymeric hydrophobic resin allowed the reaction concentration to be increased from 6 to 40 g/L, with an overall productivity of 75 g/L/day. In cases as just described, the properties of the reactant and the product must be such that the reactant can be easily desorbed from the adsorbent resin and the product easily adsorbed back onto the resin. The limitations of this method would depend upon the affinity the reactant and product have for the resin.[12] Further whole cell examples using this approach are now forthcoming,[17,18] but the results presented here may additionally provide options for sufficiently stable isolated enzymes.

This final analysis of the optimal reactor configuration will be dependent upon the properties of the reactants, products and enzymes, but significant productivity enhancements may be possible.

4. Experimental

4.1. Analytical

HPLC (Gilson, Middletown, WI, USA) separation was achieved on a Zorbax RX-C8 column (ManMod Analytical, Chadds Ford, PA, USA) by isocratic elution using a 50% acetonitrile and 50% acidified water (0.1% H3PO4) mobile phase at 1.5 mL/min. Tetralone and tetralol had retention times of 4.70 and 6.20 min, respectively. Detection was by UV at 220 nm.

4.2. Production of tetralone reductase

Tetralone reductase was isolated by centrifugation and homogenisation from cells of *T. capitatum* (MY 1890) (kindly donated by Merck Research Laboratories, Rahway, NJ, USA). Subsequent purification was through Q sepharose, hydroxy apatite and toyopearl chromatography columns.[6]

4.3. Immobilisation of tetralone reductase

Dry Eupergit C (Röhm, Darmstadt, Germany) beads (1 g) were mixed with tetralone reductase solution (5 mL) in 1 M potassium phosphate buffer pH 7.5 and left at room temperature for 72 h.

4.4. Resin screen for NADH binding

0.5 g/L NAD(H) solution dissolved in water (2 mL) was added to resin (0.2 g) (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, XAD L-323, XAD 2010, or XAD L-493) (Supelco, Poole, Dorset, UK). The suspensions were shaken continuously for 30 min and then filtered using Puradisc™ syringe filters to remove the resin (Whatman, Maidstone, Kent, UK). The remaining NADH in solution was assayed by spectrophotometer at 340 nm.

4.5. Resin screen for protein binding

XAD resin (1 g) (XAD 4, XAD 7HP, Dowex Optipore 285, XAD 1180, XAD 2000, XAD L-323, XAD 2010, or XAD L-493) (Supelco, Poole, Dorset, UK) was added to 0.2 g/L tetralone reductase solution (2 mL). After 30 min of continuous shaking, the samples were filtered using Puradisc™ syringe filters to remove the resin. The filtered samples were then assayed for total protein using the Bradford spectrophotometric assay.[19]

4.6. Binding of 6-bromo-β-tetralol on XAD L-323

Solutions of water (5×10 mL) containing 100 g/L 6-bromo-β-tetralone solution (100 μL) in methoxyethanol (final concentration 1 g/L), were added to 0.05, 0.1, 0.2, 0.4 or 0.6 g of XAD L-323. The samples were shaken continuously for 30 min. Subsequently, samples were filtered using Puradisc™ syringe filters to remove the resin and the filtrate assayed by HPLC for 6-bromo-β-tetralol.

4.7. Binding of 6-bromo-β-tetralone on XAD L-323

Solutions of water (5×10 mL) containing 100 g/L 6-bromo-β-tetralol in methoxyethanol (100 μL) (final concentration 1 g/L), were added to 0.05, 0.1, 0.2, 0.4 and 0.6 g of XAD L-323. The samples were shaken continuously for 30 min. Subsequently, samples were filtered using Puradisc™ syringe filters to remove the resin and the filtrate assayed by HPLC for 6-bromo-β-tetralone.

4.8. Bioconversion

Formate (8 mg), NAD (0.43 mg) and FDH (8 mg) were dissolved in the tetralone reductase solution (2970, 2850 and 2700 μL). The reactions were initiated through the addition of a 100 g/L tetralone solution in 30, 150 and 300 μL (final concentrations 1, 5 and 10 g/L, respectively). Formate was added in excess. The reactions were monitored over time by HPLC analysis for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.9. Bioconversion with solid phase product removal 1

Tetralone reductase (10 mL) was added to NAD (1.43 mg), FDH (20 mg) and formate (4.5 mg). The reaction was initiated with 100 g/L tetralone in methoxyethanol (100 μL) of a (final reaction concentration 1 g/L) solution. To assay the reaction mix, 200 μL samples were taken (a total of 1 mL of the reaction mixture was removed for assaying). XAD L-323 (0.5 g) was added to the reaction components to facilitate product removal. The reaction components were then filtered using a syringe filter, into a new sterile Sterilin™ reaction vessel (Bibby Sterilin, Stone, Staffs, UK). A sample of the filtered mix was assayed to check that the product and remaining substrates had been removed. The reaction was assayed at defined time points by HPLC for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

4.10. Bioconversion with solid phase product removal 2

Tetralone reductase (1 mL in total) was removed in the assaying of the first reaction. Extra tetralone reductase (1 mL) was added to the filtered reaction components with formate (4 mg), FDH (2 mg) and NAD (0.143 mg). The reaction was initiated again with 100 g/L 6-bromo-β-tetralone in methoxyethanol (100 μL). The reaction was assayed at defined time points by HPLC for 6-bromo-β-tetralone and 6-bromo-β-tetralol.

Acknowledgements

The authors are grateful to the BBSRC and Merck and Co for support of this programme. The authors are also grateful to Helen E. M. Law for help in the preparation of the manuscript.

References and notes

1. Straathof, A. J. J.; Panke, S.; Schmid, A. *Curr. Opin. Biotechnol.* **2002**, *13*, 548–556.
2. Schmid, A.; Hollmann, F.; Park, J. B.; Buhler, B. *Curr. Opin. Biotechnol.* **2002**, *13*, 359–366.
3. Devaux-Basseguy, R.; Bergel, A.; Comtat, M. *Enzyme Microbiol. Technol.* **1997**, *20*, 248–258.
4. Hummel, W. *Trends Biotechnol.* **1999**, *17*, 487–492.
5. Reddy, J.; Tschaen, D.; Shi, Y.; Pecore, V.; Katz, L.; Greasham, R.; Chartrain, M. *J. Ferment. Bioengng* **1996**, *81*, 304–309.
6. Shorrock, V. J. PhD Thesis. University of London, 2003.
7. Tschaen, D. M.; Abramson, L.; Cai, D.; Desmond, R.; Dolling, U.; Frey, L.; Karaday, S.; Shi, Y.; Verhoeren, T. R. *J. Org. Chem.* **1995**, *60*, 4324–4330.
8. Chenault, H. K.; Simon, E. S.; Whitesides, G. M. *Biotechnol. Genet. Engng Rev.* **1988**, *6*, 221–270.
9. Weuster-Botz, D.; Paschold, H.; Striegel, B.; Gieren, H.; Kula, M.-R.; Wandrey, C. *Chem. Engng Technol.* **1994**, *17*, 131–137.
10. Wichmann, R.; Wandrey, C.; Buckmann, A. F.; Kula, M.-R. *Biotechnol. Bioengng* **1981**, *23*, 2789–2802.
11. Lye, G. J.; Woodley, J. M. *Trends Biotechnol.* **1999**, *17*, 395–402.
12. Straathof, A. J. J. *J. Biotechnol. Prog.* **2003**, *19*, 755–762.
13. Lilly, M. D.; Woodley, J. M. *J. Indian Microbiol.* **1996**, *17*, 24–29.
14. D'Arrigo, P.; Fantoni, G. P.; Servi, S.; Strini, A. *Tetrahedron: Asymmetry* **1997**, *8*, 2375–2375.
15. Vicenzi, J. T.; Zmijewski, M. J.; Reinhard, M. R.; Landen, B. E.; Muth, W. L.; Marler, P. G. *Enzyme Microbiol. Technol.* **1997**, *20*, 494.
16. D'Arrigo, P.; Fuganti, C.; Fantoni, G. P.; Servi, S. *Tetrahedron* **1998**, *54*, 15017.
17. Simpson, H. D.; Alphand, V.; Furstoss, R. *J. Mol. Catal. B: Enzymatic* **2001**, *16*, 101–108.
18. Houng, J.-Y.; Liau, J.-S. *Biotechnol. Lett.* **2003**, *25*, 17.
19. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248.