Downloaded from orbit.dtu.dk on: Apr 17, 2021

DTU Library

# Application of in situ product-removal techniques to biocatalytic processes

**Lye, GJ; Woodley, John**

*Published in:*
Trends in Biotechnology

*Link to article, DOI:*
10.1016/S0167-7799(99)01351-7

*Publication date:*
1999

*Document Version*
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

*Citation (APA):*
Lye, GJ., & Woodley, J. (1999). Application of in situ product-removal techniques to biocatalytic processes. *Trends in Biotechnology*, *17*(10), 395-402. https://doi.org/10.1016/S0167-7799(99)01351-7

**General rights**
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

## References

1 Sebba, F. (1971) *J. Colloid Interface Sci.* 35, 643–646
2 Shaw, D. J. (1992) *Introduction to Colloid and Surface Chemistry* (4th edn), Butterworth–Heinemann
3 Sebba, F. (1987) *Foams and Biliquid Foams: Aphrons*, John Wiley & Sons
4 Sebba, F. (1985) *Chem. Ind.* 3, 91–92
5 Matsushita, K., Mollah, A. H., Stuckey, D. C., Del Cerro, C. and Bailey, A. I. (1992) *Colloid Surf.* 69, 65–72
6 Chapalkar, P. G., Valsaraj, K. T. and Roy, D. (1993) *Sep. Sci. Technol.* 28, 1287–1302
7 Save, S. V. and Pangarkar, V. G. (1994) *Chem. Eng. Commun.* 127, 35–53
8 Subramaniam, M. B., Blakebrough, N. and Hashim, M. A. (1990) *J. Chem. Technol. Biotechnol.* 48, 41–60
9 Jauregi, P., Gilmour, S. and Varley, J. (1997) *Chem. Eng. J.* 65, 1–11
10 Save, S. V., Pangarkar, V. G. and Kumar, S. V. (1995) *J. Chem. Technol. Biotechnol.* 62, 192–199
11 Jauregi, P. and Varley, J. (1998) *Biotechnol. Bioeng.* 59, 471–481
12 Noble, M., Brown, A., Jauregi, P., Kaul, A. and Varley, J. (1998) *J. Chromatogr. B* 711, 31–43
13 Krijgman, J. and Jenkins, R. O. (1992) *Product Recovery in Bioprocess Technology*, Butterworth–Heinemann
14 Kaster, J. A., Michelsen, D. L. and Velander, W. H. (1990) *Appl. Biochem. Biotechnol.* 24/25, 469–484
15 Bredwell, M. D., Telgenhoff, M. D. and Worden, R. M. (1995) *Appl. Biochem. Biotechnol.* 52/52, 501–509
16 Jackson, A., Kommalapati, R., Roy, D. and Pardue, J. (1998) *J. Environ. Sci. Health. Part A Environ. Sci. Eng.* 33, 369–384
17 Amiri, M. C. and Woodburn, E. T. (1990) *Trans. Inst. Chem. Eng.* 68A, 154–160
18 Ciriello, S., Barnett, S. M. and Deluise, F. J. (1982) *Sep. Sci. Technol.* 17, 521–534
19 Cilliers, J. J. and Bradshaw, D. J. (1996) *Miner. Eng.* 9, 235–241
20 Caballero, M., Cela, R. and Perez-Bustamante, J. A. (1989) *Sep. Sci. Technol.* 24**,** 629–640
21 Roy, D., Valsaraj, K. T. and Kottai, S. A. (1992) *Sep. Sci. Technol.* 25, 573–588
22 De Vries, A. J. (1972) in *Adsorptive Bubble Separation Techniques* (Lemlich, R., ed.), pp. 7–31, Academic Press
23 Tatterson, G. B. (1991) in *Fluid Mixing and Gas Dispersion in Agitated Tanks* (Tatterson, G. B., ed.), pp. 417–523, McGraw-Hill
24 Kommalapati, R. R., Roy, D., Valsaraj, K. T. and Constant, W. D. (1996) *Sep. Sci.Technol.* 31, 2317–2333
25 Pinfold, T. A. (1972) in *Adsorptive Bubble Separation Techniques* (Lemlich, R., ed.), pp. 75–89, Academic Press
26 Chapalkar, P. G., Valsaraj, K. T. and Roy, D. (1994) *Sep. Sci. Technol.* 29, 907–921
27 Hashim, M. A. and Sengupta, B. (1998) *Bioresource Technol.* 64, 199–204
28 Wallis, D. A., Michelsen, D. L., Sebba, F., Carpenter, J. K. and Houle, D. (1985) *Biotechnol. Bioeng. Symp.* 15**,** 399–408
29 Hashim, M. A., Sengupta, B. and Subramaniam, M. B. (1995) *Bioseparation* 5, 167–173
30 Hashim, M. A., Sengupta, B. and Kumar, S. V. (1995) *Biotechnol. Tech.* 9, 403–408
31 Hashim, M. A., Sengupta, B., Kumar, S. V., Lim, R., Lim, S. E. and Tan, C. C. (1998) *J. Chem. Technol. Biotechnol.* 71, 335–339
32 Karger, B. L. (1972) in *Adsorptive Bubble Separation Techniques* (Lemlich, R., ed.), pp. 145–154, Academic Press
33 Save, S. V., Pangarkar, V. G. and Kumar, S. V. (1993) *Biotechnol. Bioeng.* 41, 72–78
34 Bredwell, M. D. and Worden, R. M. (1998) *Biotechnol. Prog.* 14, 31–38
35 Worden, R. M. and Bredwell, M. D. (1998) *Biotechnol. Prog.* 14, 39–46


# Application of *in situ* product-removal techniques to biocatalytic processes

Gary J. Lye and John M. Woodley

Biocatalytic processes for the manufacture of small, highly functionalized molecules frequently have limited productivity. A common reason for this is the presence of the reaction products that can cause inhibitory or toxic effects (making poor use of the enzyme) or promote unfavourable equilibria (giving low conversions). In each case, the product needs to be removed as soon as it is formed in order to overcome these constraints and hence increase the productivity of the biocatalytic process. Here, we review the need for *in situ* product removal and the process research required for its implementation.

In response to mounting socio-economic pressure, national governments are placing increasing demands on pharmaceutical companies to produce better medicines at lower prices. 'Better' medicines are those with a higher specificity and fewer side effects, which inevitably means that the biologically active molecules to be manufactured are structurally complex. In 1997, for example, 50 of the top 100 drugs were single isomers[1]; drugs now reaching the marketplace possess multiple chiral centres[2].

As a result of these demands on pharmaceutical companies, biocatalysis is now receiving considerable attention for the synthesis of molecules involved in the treatment of human immunodeficiency virus (HIV), heart disease, cancer, diabetes and bacterial infections including tuberculosis[3]. Similarly, in order to drive drug prices down, the product synthesis and purification sequences

*G. J. Lye (g.lye@ucl.ac.uk) and J. M. Woodley (j.woodley@ucl.ac.uk) are at the Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London, UK WC1E 7JE.*

 0167-7799/99/$ – see front matter © 1999 Elsevier Science Ltd. All rights reserved. PII: S0167-7799(99)01351-7 

**Box 1. Quantifying the benefits of ISPR**

Before implementing *in situ* product-removal (ISPR) methodologies, the benefits that are required to offset the increased costs in terms of process R&D and capital equipment must be clearly defined. It is also necessary to do this quantitatively so that different ISPR methodologies can be compared on the same basis. The potential benefits of ISPR and their impact on the design and operation of biocatalytic processes are summarized in Table I.

The basis chosen for the comparison will be specific to a particular process. In cases where catalyst costs are high, for example, it will be necessary to make the most effective use of the catalyst, in which case the yield of product on catalyst ($g_P\ g_B^{-1}$) is important. In other cases, such as the transformation of an expensive pharmaceutical intermediate, the yield of product from substrate ($g_P\ g_S^{-1}$) might be the preferred basis.

**Table I. The potential benefits of *in situ* product removal**

| Benefit | Basis | Impact |
|---|---|---|
| Increased product concentration | $g_P\ l^{-1}$ | Reduced reactor volume, easier DSP |
| Increased yield on biocatalyst | $g_P\ g_B^{-1}$ | Reduced catalyst costs |
| Increased yield on substrate | $g_P\ g_S^{-1}$ | Reduced substrate costs |
| Increased volumetric productivity | $g_P\ l^{-1}\ h^{-1}$ | Reduced reactor volume and/or processing time, easier DSP |

Abbreviations: DSP, downstream processing; $g_B$, grams biocatalyst; $g_P$, grams product; $g_S$, grams substrate.

must have greater overall volumetric productivities (and/or yields) and the process research and development must be completed more quickly in order to enter the market as early as possible[4].

The application of enzyme-based catalysts is moving from racemic resolutions to asymmetric syntheses in order to improve yields and to allow the production of more-complex molecules. Similarly, bioreactor operation is moving from dilute to high concentrations of reactant and product in order to meet the volumetric productivities required in the industrial context. Although many biocatalytic processes (whole-cell and isolated-enzyme mediated) are now finding applications in industry, there remain three fundamental barriers to their widespread use. First, cheap, stable enzyme catalysts must become more easily available. Second, systematic design methods are needed to speed up the development of whole process sequences and to enable rational choices to be made between chemical and biocatalytic routes to the same end product. Finally, the low productivities that are often observed with biocatalytic systems, relative to the chemical equivalent, must be enhanced in order to develop more-economic processes. It is the last of these barriers and, in particular, the potential of *in situ* product removal (ISPR) to enhance bioreactor productivities that is the focus of this article.

## Limitations of biocatalytic processes and the role of ISPR

The productivity of biocatalytic processes is frequently limited by the need to operate the reaction under conditions unsuited to the biocatalyst. Indeed, this differentiates biological from chemical catalysis, in that the optimal environment for the biological catalyst has been carefully evolved for operation under natural physiological conditions, whereas chemical catalysts are designed for a specific conversion at user-defined conditions. Inevitably, the need for high process productivities outside the environment for which the biocatalyst was evolved leads to compromises over bioreactor design and operation. In several cases, however, compromises are not sufficient and it is necessary to maintain one environment around the biocatalyst while the bulk of the reactor operates under different conditions.

This philosophy of compartmentalization is, in principle, an effective strategy to overcome the problem of low productivities. It has already been successfully applied to cases where the pH[5] or substrate concentration[6] need to be different for the reactor and the biocatalyst. A further need is for compartmentalization of the product owing to inhibitory (reversible loss of catalytic activity) or toxic (irreversible loss of catalytic activity) effects on the biocatalyst, to product degradation and/or to unfavourable reaction equilibria. These limitations to productivity in the presence of the product have been well documented in the past[7,8].

ISPR methods address these limitations by selectively removing the product from the vicinity of the biocatalyst as soon as it is formed and can also provide further benefits for the subsequent downstream processing[9–11]. ISPR methods can increase the productivity or yield of a given biocatalytic reaction by any of the following means[12]: (1) overcoming inhibitory or toxic effects; (2) shifting unfavourable reaction equilibria; (3) minimizing product losses owing to degradation or uncontrolled release; and (4) reducing the total number of downstream-processing steps. The various bases for quantitative comparison of ISPR techniques and the impact these can have on the design and operation of a biocatalytic process are summarized in Box 1.

Despite these advantages, however, the application of ISPR to the production of small molecules has been slow except in a limited number of well-known cases. The primary examples are of low-value, high-volume products, such as the removal of organic acids and solvents from fermentation processes[9]. In this article, we explore some of the reasons for this and outline the latest methods available for the selective *in situ* recovery of high-value, low-volume products from biocatalytic processes, so that the potential of the technology can be fully exploited.

## The separations challenge

Several separation steps are necessary in a biotransformation process to recover and purify the desired product (Fig. 1). The ease or otherwise of the separation will depend upon the type of reaction being performed, the biocatalyst form (whole cell, immobilized enzyme or free enzyme) and the design and operation of the bioreactor. These factors will determine whether or not there are undesirable regio- or stereoisomers of the product to be removed, whether or not there are compounds resulting from the decomposition or further reaction of the product that need to be separated and, ultimately, the relative concentrations of substrate and product molecules in the reactor at the end of the reaction. The last point is particularly important, because the substrate and product molecules of a biocatalytic process will generally be structurally and chemically very similar to each other and hence extremely difficult to separate.

In terms of the sequence in which the various separation steps are carried out, the 'rule of thumb' is generally to perform first those operations for which the separation driving force is largest; that is, where there is the greatest difference in physicochemical properties between the species to be separated. Given the large size difference between the biocatalyst and substrate and product molecules, especially when using immobilized-enzyme or whole-cell biocatalysts, the first separation step will logically be some form of solid–liquid separation such as filtration or centrifugation.

There are various physical and chemical methods for the subsequent separation of substrate and product molecules, and these can be classified according to the basis on which the separation is achieved (Table 1). The most appropriate techniques are those that involve partitioning of the product into a second liquid phase or the adsorption onto or generation of a solid phase. Although examples of product recovery using distillation do exist, even with *in situ* applications[13,39], such techniques are often not sufficiently selective and are only applicable to limited classes of biocatalytic process, such as those with small and highly volatile molecules. The selectivity and capacity requirements of the various separation techniques will be discussed later.

The mode of operation of ISPR techniques (i.e. whether they are batch, fed-batch or continuous and whether the separation step occurs within the bioreactor or outside it) have been extensively discussed in previous reviews on this topic[9,12] and will not be repeated here. Much of the work reported was, however, concerned with the recovery of ethanol or organic acids from fermentation processes. Other reviews on process integration have been confined to a particular class of

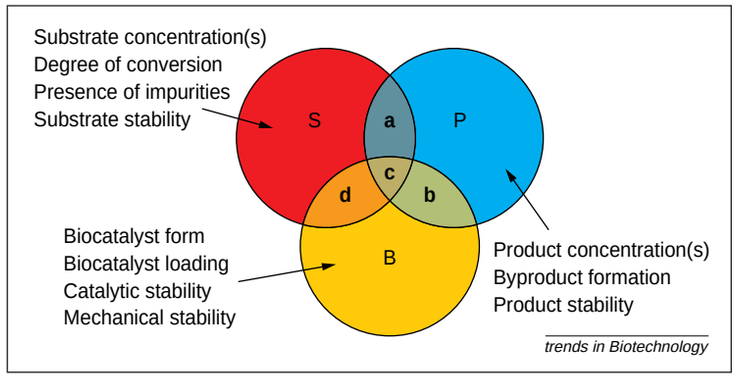


**Figure 1**

Schematic representation of the separation challenges to be addressed in the design of a biocatalytic process. The circles represent the three components present in the reactor – substrate (S), product (P) and biocatalyst (B). The intersections of the circles represent the four permutations of separations required: (**a**) P from S; (**b**) P from B; (**c**) P from S and B; and (**d**) S from B. The adoption of an *in-situ*-product-retrieval technique requires that separations **a**, **b** and **c** are carried out simultaneously; separation **d** is necessary to facilitate reuse of either the biocatalyst or unconverted substrate. The labels on each circle represent the key factors affecting the ease of separation of that component from any other.

**Table 1. Classification of key substrate and product separation techniques according to the basis of separation**

| Separation basis (driving force) | Comments | Example techniques | Refs[a] |
|---|---|---|---|
| **Physical properties** | | | |
| Volatility | Few examples with these properties. | Distillation | 13 |
| | | Gas stripping | 14 |
| Molecular weight or size | The difference between substrate and product is frequently too small. | Membranes (MF, UF etc.) | 15 |
| | | Centrifugation | |
| | | Size exclusion | |
| | | Pervaporation or perstraction[b] | 15–20 |
| Solubility | High capacity but generally low selectivity. | Extraction, ATPS, $SCCO_2$ | 21–26 |
| | | Precipitation | 27 |
| | | Crystallization | |
| **Chemical properties** | | | |
| Charge | High selectivity but generally low capacity. | Ion-exchange, electrodialysis[b] | 24,28,29 |
| Hydrophobicity | | HIC, adsorption | 30–37 |
| Specific elements | | Reversible complexation | 34 |
| | | Affinity methods | 38 |

[a]Examples of use for *in situ* product-removal applications only.
[b]Certain techniques are based on a combination of separation driving forces.
Abbreviations: ATPS, aqueous two-phase systems; HIC, hydrophobic-interaction chromatography; MF, microfiltration; $SCCO_2$, supercritical carbon dioxide; UF, ultrafiltration.

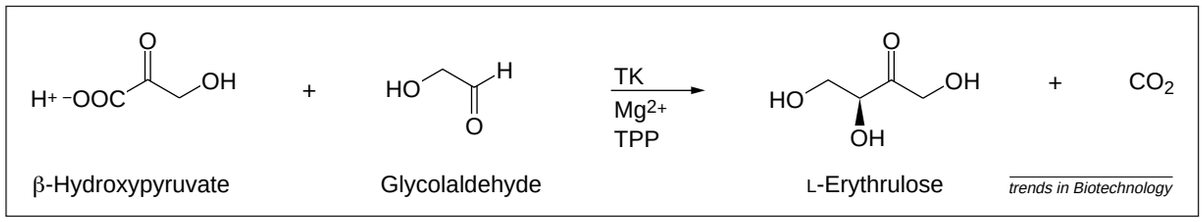


**Figure 2**

Transketolase-catalysed condensation of glycolaldehyde with β-hydroxypyruvate to yield L-erythrulose with the evolution of carbon dioxide. Abbreviations: TK, transketolase; TPP, thiamine pyrophosphate.

biocatalytic reaction, such as those involving lipases[39]. The following examples show how some of the recovery and separation techniques listed in Table 1 have been applied to a range of biocatalytic processes, with varying degrees of success and using different ISPR configurations.

## Examples of ISPR application

### *Synthesis of L-erythrulose with transketolase*

Enzymes have proved to be particularly useful catalysts for asymmetric carbon–carbon-bond synthesis. Useful reactions can be catalysed by lyases (including aldolases) and transferases [e.g. transketolase (TK; EC 2.2.1.1)]. We have used TK (isolated from recombinant *Escherichia coli* in which the enzyme was overexpressed) to condense β-hydroxypyruvate (ketol donor) with glycolaldehyde (aldehyde acceptor), yielding L-erythrulose (Fig. 2). L-Erythrulose inhibits the enzyme, reducing the rate of the reaction, thus providing a clear logic for the application of ISPR[40].

Using this condensation reaction as a model system, a variety of methods have been investigated as ISPR techniques, including ion exchange, complex formation and physical adsorption. Complex formation based on phenylboronate–diol interactions showed the greatest potential for use as a selective means of removing L-erythrulose from the reaction medium. Because concentrations of free phenylboric acid of $\geqslant$100 mM were toxic to TK, an immobilized phenylboronate resin (Affigel 601) was used[34]. The experimentally determined binding capacities indicated that, with L-erythrulose, β-hydroxypyruvate and glycolaldehyde present, no β-hydroxypyruvate bound but that 21% (on a molar basis) of glycolaldehyde bound together with L-erythrulose[34]. Substrate feeding of the glycolaldehyde was subsequently used to overcome the low selectivity of these adsorbents[41] and the TK was immobilized to aid separation of the biocatalyst from the product[42]. When ISPR was performed on the model reaction using this resin with substrate feeding, the reaction proceeded to completion[34]. Further work is required to assess the real benefits of ISPR in this specific case. Clearly, the use of this technique will have applications in the recovery of products other than L-erythrulose, because α-hydroxylated aldehydes will always yield products containing a *cis*-diol moiety.

### *Synthesis of fluorocatechol by* Pseudomonas putida *ML2*

The specific hydroxylation of aromatic compounds such as benzene or toluene to their corresponding *cis* glycol involves the first enzyme of the aromatic degradation pathway, benzene dioxygenase. It is also possible to make use of the second enzyme in the pathway, a dehydrogenase, to produce the corresponding catechol, which, in a bacterium, is normally degraded further. However, using fluorobenzene as the reactant, the product formed (fluorocatechol) is not attacked by the third enzyme, catechol 1,2-dioxygenase, and so the fluorocatechol accumulates. This compound is a valuable precursor for the synthesis of pharmaceuticals such as adrenergic catecholamines and biogenic amines. This reaction [oxidation of fluorobenzene to fluorocatechol by *P. putida* ML2 (Fig. 3)] has been used as a model system to study several process constraints[43].

The high toxicity and low aqueous solubility of the reactant fluorobenzene caused difficulties that were overcome by controlled feeding of the reactant, thus maintaining a low concentration within the reactor[33]. In addition, fluorocatechol is toxic to the microorganism at concentrations $>0.2\ g\ l^{-1}$. To alleviate this toxicity, ISPR was implemented via adsorption onto activated carbon in an external packed bed. With this material, fluorocatechol binding was unaffected by the presence of low concentrations of fluorobenzene and a high degree of selective binding was not necessary owing to the controlled feeding of the substrate. As a result of implementing ISPR, a 30-fold improvement in final product concentration was achieved upon elution from the adsorbent[33].

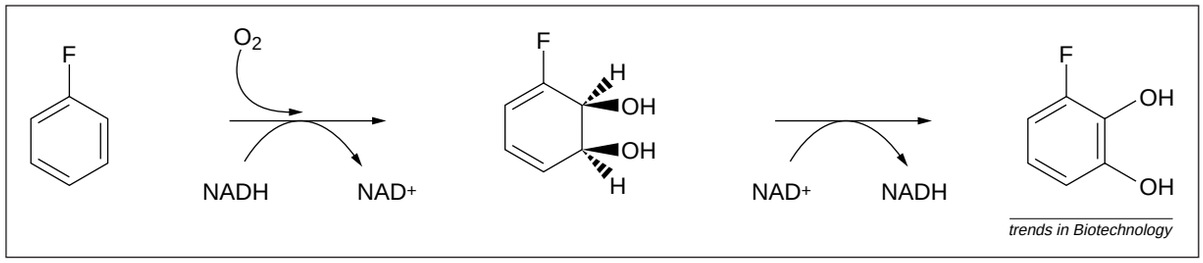


**Figure 3**

Two-step oxidation of fluorobenzene to fluorocatechol by *Pseudomonas putida* ML2.

**Table 2. Examples of industrial *in situ* product removal research**

| | Stereoselective reduction[30] | Monoterpene synthesis[32] | 6-Hydroxynicotinic-acid synthesis[27] |
|---|---|---|---|
| **ISPR rationale** | Toxicity | Inhibition | Inhibition |
| **ISPR method** | Amberlite XAD-7 | Amberlite XAD-2 | Continuous precipitation |
| **Biocatalyst** | *Zygosaccharomyces rouxii* | *Ceratocystis variospora* | *Achromobacter xylosoxidans* |
| **Scale** | 300 l | 20 l | Not stated |
| **Benefit** | 6.7× increase in product concentration | 48× increase in product concentration | Increased volumetric productivity |

Abbreviation: ISPR, *in situ* product removal.

***Industrial examples of ISPR***

Although many companies are actively involved in biocatalysis for the production of fine chemicals, agrochemicals and pharmaceuticals[3], the majority of industrial processes are still based on sequential unit operations. Industrial examples of research into ISPR techniques do exist, however, for a range of reactions and have produced some impressive results (Table 2). In all cases, these have been implemented to overcome inhibitory or toxic effects of the product on the biocatalyst. In many cases, ISPR was implemented together with controlled feeding or release of the substrate, which was also toxic to the biocatalyst, to maintain low substrate concentrations in the bioreactor medium. The separation techniques involved generally rely on adsorption to hydrophobic resins or on liquid–liquid extraction, again suggesting that these are the most appropriate separation technologies for ISPR applications (Table 1).

The hydrophobic Amberlite XAD resins have been the most widely used in the industrial context. The stereoselective reduction of 3,4-methylene dioxyphenyl acetone to *S*-3,4-methylene-dioxyphenyl isopropanol by a whole-cell *Zygosaccharomyces rouxii* biocatalyst has been demonstrated up to a scale of 300 l using an agitated filter unit as a bioreactor[30]. Preadsorption of the substrate onto an XAD-7 resin allowed simultaneous *in situ* substrate supply and ISPR, so that the final product concentration (after elution) in a batch process increased from 6 g $l^{-1}$ to 40 g $l^{-1}$. The maximum capacity of the XAD-7 for *S*-3,4-methylenedioxyphenyl isopropanol was approximately 80 g $l^{-1}$ of wet resin. Similarly, XAD-2 was used to enhance the production of monoterpenes, such as geraniol, that inhibit a strain of *Ceratocystis variospora* used for their production by fermentative bioconversion[32]. Here, on the 20 l scale, the use of ISPR increased the final product concentration from 0.04 g $l^{-1}$ to 1.9 g $l^{-1}$.

Jaquet and co-workers discussed the selection of an ISPR technique for the recovery of 3-pyridylacetic acid from the whole-cell *Pseudomonas-oleovorans*-catalysed transformation of 3-ethylpyridine[24]. Reactive solvent extraction with Aliquat 336 in octan-1-ol was the ISPR technique finally selected; simultaneous extraction and back-extraction of the acid product were achieved in a membrane-based contactor, allowing final product concentrations >15 g $l^{-1}$. One example of a continuous ISPR process has also been described for the conversion of niacin to 6-hydroxynicotinic acid by whole *Achromobacter xylosoxidans* LK1 cells[27]. This process was developed because the magnesium salt of the product, but not of the substrate, is insoluble at neutral pH. A continuous process using a stirred-tank reactor and integrated settling device for recovery of the product crystals allowed productivities of 1.5 g $l^{-1}$ $h^{-1}$ to be obtained.

Finally an ISPR process operated commercially for the lipase-mediated resolution of (±)-*trans*-methyl-methoxyphenylglycidate to produce an intermediate in the synthesis of Diltiazem has recently been reported[20]. The rationale for operating in ISPR mode was that the hydrolysis product, (2*S*,3*R*)-methoxyphenylglycidic acid, rapidly decomposed in the reactor medium to 4-methoxyphenylacetaldehyde, which proved to be an irreversible inhibitor of the enzyme. The industrial process[20], based on an extractive hollow-fibre membrane reactor with an effective membrane area of 1440 $m^2$, has now been operated by Tanabe in Japan for four years at an overall productivity of 75 kg $m^{-2}$ $yr^{-1}$.

## New operations for ISPR applications

***Requirements for new separation techniques***

As outlined in the above examples, one of the major requirements for ISPR is the need to improve the selectivity of the separation technique employed; that is, to discriminate more effectively between the substrate and product molecules (Fig. 4). The operating conditions for the majority of reported ISPR applications have involved a product concentration similar to or significantly greater than the concentration of unreacted substrate (Fig. 4b,c). In general, as illustrated above, this can be facilitated by controlled feeding of the substrate. However, many reactions of interest (e.g. oligosaccharide synthesis) must, owing to unfavourable reaction equilibria, be operated in the region where the substrate concentration is greater than that of the product (Fig. 4a). Here, the substrate must be present in a large excess to shift the equilibrium position of the reaction towards product formation. Separation techniques to be used in these instances must obviously exhibit highly selective binding of the product and low nonspecific binding of the substrate.

Equally, for the development of economic processes, the capital and operating costs of the ISPR technique must be minimized. In practice, this means that the separation technique must have a high capacity for the target molecule (on a mass basis) to reduce the quantities of adsorbents, complexing agents and solvents required, as well as exhibiting the required selectivity. Although extractive processes generally have a high capacity for the target compound, research needs to be directed towards

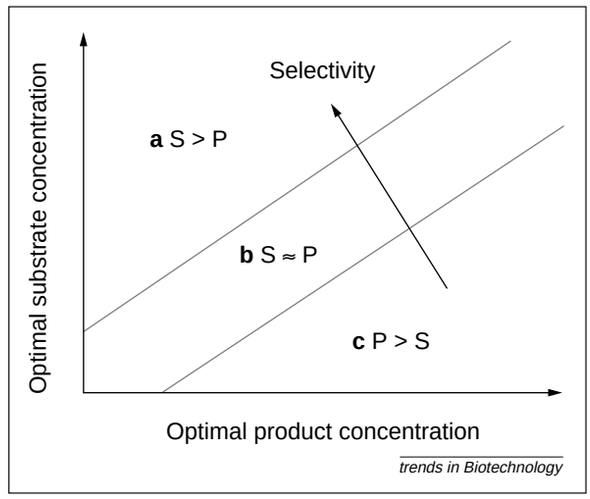


**Figure 4**

Selectivity requirements for *in situ* product removal (ISPR) separation techniques. The diagram schematically plots the optimal substrate (S) and product (P) concentrations required for effective use of the biocatalyst and reaction components. Three regions can be distinguished: (**a**) where the optimal substrate concentration is in excess of the product (S > P); (**b**) where substrate and product concentrations are very similar (S ≈ P); and (**c**) where the product concentration is in excess of the substrate (P > S). Movement from **c** to **a** requires the implementation of increasingly selective ISPR techniques if the product is to be separated from the substrate under the conditions present in the reactor.

increasing the selectivity of such processes. Likewise, for separation techniques based on adsorption or complexation, there is a need for resins with increased capacity.

For example, using Affigel 601 to recover L-erythrulose in TK-catalysed condensations, experimentally determined capacities of up to 0.66 mmoles (g resin)$^{-1}$ were measured in model solutions. Under experimental ISPR conditions, however, capacities of only 0.4 mmoles (g resin)$^{-1}$ were observed because of the ionic strength of the medium (R. P. Chauhan, PhD thesis, University of London, London, UK, 1996). This indicates that at least a tenfold increase in capacity would be required for this reaction to operate effectively with ISPR on a reasonable scale.

Novel separation techniques to meet these requirements for ISPR applications will emerge via advances in either the underlying science (to generate new, more-selective techniques) or in the process engineering (to enhance the performance of existing operations). The most attractive separation techniques, particularly in the industrial context, will tend to be those that can be applied to the widest range of applications because they are generic or can be made so with little modification to the design and operation of the existing process equipment. Two such recent advances, molecularly imprinted polymers (MIPs) and counter-current chromatography (CCC), have the potential to be applied as highly selective ISPR tools.

### *Molecularly imprinted polymeric adsorbents*

Molecular imprinting is an emerging technique in which polymeric adsorbents are synthesized that exhibit highly selective binding for a particular target molecule. Current imprinting strategies rely on either covalent or noncovalent interactions between the target molecule and the polymer during the imprinting and adsorption stages, or on a combination of both[44–46]. The noncovalent approach is the easiest and most widely used methodology to date (Box 2). MIPs have been shown to discriminate between stereoisomers and between different molecules on the basis of a single hydroxyl group[46], and are therefore of interest as a highly selective ISPR tool. An added benefit is that molecular imprinting is a potentially generic separation technique, as shown by the wide range of product categories (e.g. pharmaceuticals, pesticides, amino acids, carbohydrates, dyes and metal ions) to which it has been successfully applied[45].

The known characteristics of MIPs can be compared with the desired criteria of solid adsorbents for use in ISPR applications (Table 3). Although the selectivity and mechanical and chemical stability of the polymers are excellent, their major drawback is currently their low capacity for the target molecule. Ongoing research on the rational design of functional monomers should improve this in the near future. A tenfold increase in capacity, together with the intensive application and reuse of the adsorbents, might lead to their eventual introduction in industrial processes.

MIPs have recently been used as an ISPR tool to overcome an unfavourable reaction equilibrium in the synthesis of the artificial sweetener aspartame[38]. Enzymatic condensations of benzyloxycarbonyl-L-aspartic acid (Z-L-Asp) with L-phenylalanine methyl ester (L-Phe-OMe) carried out in the presence of aspartame-imprinted polymers increased the yield of aspartame by a factor of more than four.

### *Counter-current chromatography*

CCC is a form of chromatography using two immiscible liquid phases (rather than a conventional solid-phase ligand support) to separate solutes on the basis of their relative solubility in the two solvents[47]. Solvent pairs can be aqueous–aqueous, aqueous–organic or organic–organic. The technique, which is essentially an intensive liquid–liquid extraction process, allows chromatographic-quality separations but with a much greater capacity than conventional solid adsorbents. It can be applied to a wide range of purification applications, including natural-product isolations (e.g. antibiotics and metabolic intermediates) and fine-chemical separations. As with MIPs, it is a potentially generic separation technique: by modifying the relative polarity of the two liquid phases, many different solute separations can be performed in the same machine.

Although there are several devices currently available, those based on the 'epicyclic coil planet centrifuge' design[47] have the greatest potential to become a generic, preparative-scale, purification operation and hence ISPR tool. The principle of operation of these devices is as follows[48]. An inert plastic or stainless steel tube (internal diameter typically 1–3 mm) is first wound around a drum. The drum is then rotated in a planetary, epicyclic motion around a stationary (or sun) gear. During the period of rotation, each point within the coil will experience an alternating acceleration field with the greatest force occurring at the furthest point from the centre of rotation. The fluctuating fields will establish alternating zones of phase mixing (low acceleration

force) and phase separation (high acceleration force) of the two immiscible liquids in the coil. This repeated action of phase mixing and separation is typical of a conventional equilibrium-stage separation process except that, in this case, owing to the motion and speed of rotation of the coil, thousands of stages can occur in a single machine. Fractionation of a sample is usually achieved by pumping the less-dense mobile phase through the coil, where it is repeatedly contacted with the denser stationary phase (which is retained within the coil owing to its rotation).

The mixture to be separated is introduced into the mobile phase and the fractionated components emerge from the end of the tube in the order of their partition coefficients. The quality of fractionation that can be achieved depends upon several factors including the physical and chemical properties of the two phases (e.g. density, viscosity, polarity), the properties of the coil (e.g. construction material, internal diameter, length), the mobile-phase's flow rate and the mechanical design of the instrument (e.g. drum radius, sun-gear radius, rotational speed). The machine is also versatile in its mode of operation. Either of the two solvent phases can be used as the stationary phase, and the mobile phase can also be switched during operation so that highly retained solutes can be eluted from the coil. A key design requirement for ISPR applications of this technology will be to match the solvent characteristics for selective elution with those already established for extractive biocatalysis[49]. Unlike conventional HPLC, CCC machines can handle crude feed materials containing particulates, although phases with a tendency to emulsify can cause operational problems resulting in the stationary phase overflowing as emulsified droplets.

For ISPR applications, biological catalysts such as enzymes or even whole cells can be immobilized within the stationary phase of the coil. Provided that the product preferentially partitions to the mobile phase, it can be effectively removed from the environment of the biocatalyst as soon as it is formed. This principle has already been successfully demonstrated for the enantioselective hydrolysis of 2-cyano-cyclopropyl-1,1-dicarboxylic acid dimethyl ester[50], in which the efficient separation of product from substrate and biocatalyst in a single unit also simplified the subsequent product-purification steps. Separation specificity can also be improved in a similar manner by the dissolution of affinity ligands or chiral selection reagents in either of the two phases[51].

## Conclusions

There is a clear role for the application of ISPR techniques in improving the yield and productivity of biocatalytic processes. However, there are several limitations to the use of current separation techniques such as low selectivity, low capacity or both. New separation techniques, such as the use of MIPs and CCC, are currently being researched to overcome some of these limitations.

In addition to the study of new separation techniques, research is required in several areas to provide a more complete evaluation of the potential of ISPR. For example, ISPR needs further evaluation for reactions with unfavourable equilibria and there is a need for an economic analysis of the benefits of ISPR compared

### Box 2. The noncovalent approach to the preparation of molecularly imprinted polymers

To synthesize molecularly imprinted polymers (MIPs) using the noncovalent approach, polymerizable monomers containing functional groups complementary to the target (template) molecule must first be selected. This selection depends upon the chemical functionality of the target molecule and whether hydrogen bonding or electrostatic or hydrophobic interactions are to be exploited in the solvent used for rebinding of the target molecule. Widely used functional monomers include methacrylic acid, 4-vinylpyridine and 4-vinylbenzoic acid. The functional monomers are associated with the template molecule dissolved in an organic solvent (Fig. I), together with a cross-linking reagent such as ethylene glycol dimethacrylate or divinylbenzene, and a polymerization initiator. The association can be with a single functional monomer or with a mixture, as shown here. The functional monomers are then copolymerized, using either bulk or suspension polymerization techniques, to form a highly cross-linked, macroporous polymer. Extraction of the template molecules yields particles with well-defined three-dimensional cavities specific for the adsorption of the original target molecule in terms of size, shape and chemical functionality.

The most important parameters for selective binding to MIPs are the selection of the functional monomer(s) and its association with the template molecule in the imprinting solvent. The choice of imprinting solvent and cross-linker will also influence the porosity, internal surface area, mechanical properties and nonspecific binding of the final polymer. The mean particle size and size distribution are important from the engineering point of view, as these will determine the solute-mass-transfer kinetics and hence the best mode of application of the MIPs.

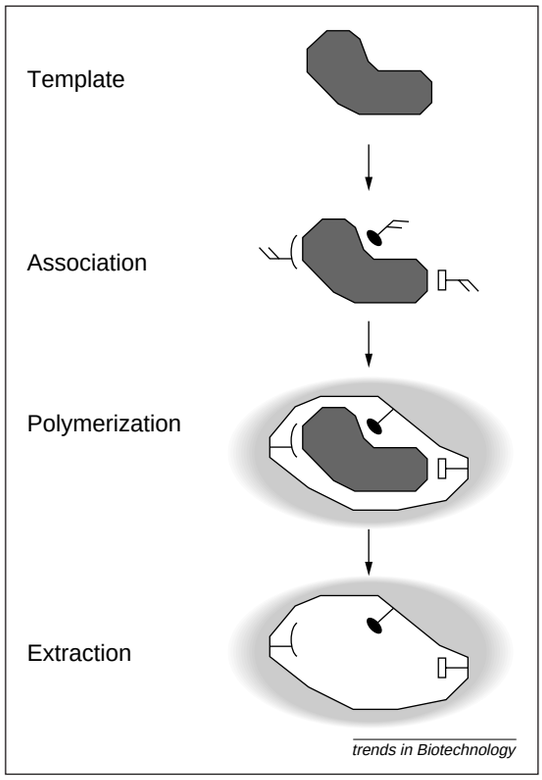


**Figure I.**

**Table 3. Selection criteria for solid adsorbents compared with those of imprinted polymers for *in situ* product-removal applications**

| Desired criteria for solid adsorbents[a] | Known characteristics of molecularly imprinted polymers[b] |
|---|---|
| High capacity for target molecule | Low capacity (<40 mg $g^{-1}$ in batch mode) |
| Favourable adsorption isotherm | Langmuir–Fraudlich isotherms |
| Adequate mass-transfer kinetics | Rapid adsorption kinetics |
| High degree of selective binding | Highly selective binding |
| Low degree of nonspecific binding | Significant nonspecific binding |
| Readily regenerated and recycled | Easy regeneration and recycling |
| Mechanical and chemical stability | Excellent mechanical and chemical stability |
| Biocompatible (toxicity, biofilms etc.) | Not known |
| Sterilizable (in certain cases) | Sterilizable |
| Reasonable cost | Initially expensive |

[a]Adapted from Ref. 7.
[b]Compiled from Ref. 45 and Lye and Stein (unpublished).

with the use of sequential downstream-processing operations. Finally, ISPR needs to be evaluated in parallel with molecular-biology techniques such as directed evolution and protein engineering[52,53], which have now shown the potential to overcome issues of product inhibition (albeit for a limited number of model enzymes). Here, the challenge will be quantitatively to evaluate the speed of implementation and potential benefit of the biological techniques with those of ISPR.

**Acknowledgments**

The Advanced Centre for Biochemical Engineering is sponsored by the UK Biotechnology and Biological Sciences Research Council and we are grateful for their support. GJL would also like to thank Esso and the Royal Academy of Engineering for the award of an Engineering Fellowship and the Nuffield Foundation for financial support (NUF-NAL).

**References**

**1** Stinson, S. C. (1998) *Chem. Eng. News* 76, 83–104
**2** Buckland, B. C. *et al.* (1999) *Metabolic Eng.* 1, 63–74
**3** McCoy, M. (1999) *Chem. Eng. News* 77, 10–14
**4** Pisano, G. P. and Wheelwright, S. C. (1995) *Harvard Bus. Rev.* 73, 93–105
**5** Byers, J. P., Shah, M. B., Fournier, R. L. and Varanasi, S. (1993) *Biotechnol. Bioeng.* 42, 410–420
**6** van den Tweel, W. J. J., de Bont, J. A. M., Vorage, M. J. A. W., Marsman, E. H., Tramper, J. and Koppejan, J. (1988) *Enzyme Microb. Technol.* 10, 134–135
**7** Mattiasson, B. and Holst, O., eds (1991) *Extractive Bioconversions*, Marcel Dekker
**8** Roffler, S. R., Blanch, H. W. and Wilke, C. R. (1984) *Trends Biotechnol.* 25, 129–136
**9** Freeman, A., Woodley, J. M. and Lilly, M. D. (1993) *Biotechnology* 11, 1007–1012
**10** Szathmary, S. and Grandics, P. (1990) *Biotechnology* 8, 924–925
**11** Daugulis, A. J. (1988) *Biotechnol. Prog.* 4, 113–122
**12** Chauhan, R. P. and Woodley, J. M. (1997) *CHEMTECH* 27, 26–30
**13** Paiva, A. L. and Malcata, F. X. (1994) *Biotechnol. Tech.* 8, 629–634
**14** Lamare, S. and Legoy, M. D. (1993) *Trends Biotechnol.* 117, 413–418
**15** Molinari, F., Aragozzini, F., Cabral, J. M. S. and Prazares, D. M. F. (1997) *Enzyme Microb. Technol.* 20, 604–611
**16** Mathys, R. G., Heinzelmann, W. and Witholt, B. (1997) *Chem. Eng. J.* 67, 191–197
**17** Lamer, T., Spinnler, H. E., Souchon, I. and Voilley, A. (1996) *Process Biochem.* 31, 533–542
**18** Doig, S. D., Boam, A. T., Leak, D. I., Livingston, A. G. and Stuckey, D. C. (1998) *Biotechnol. Bioeng.* 58, 587–594
**19** Wehtje, E., Kaur, J., Aldercreutz, P., Chand, S. and Mattiasson, B. (1997) *Enzyme Microb. Technol.* 21, 502–510
**20** Lopez, J. L. and Matson, S. L. (1997) *J. Membr. Sci.* 125, 189–211
**21** Kwon, S. J., Han, J. J. and Rhee, J. S. (1995) *Enzyme Microb. Technol.* 17, 700–704
**22** Marty, A., Combs, D. and Condoret, J. S. (1994) *Biotechnol. Bioeng.* 43, 497–504
**23** Hernandez-Justiz, O., Fernandez-Lafuente, R., Terreni, M. and Guisen, J. M. (1998) *Biotechnol. Bioeng.* 59, 73–79
**24** Jaquet, A., Marison, I. W., Meyer, H-P. and von Stockar, U. (1996) *Chimia* 50, 426–427
**25** Kaul, R. and Mattiasson, B. (1991) in *Extractive Bioconversions* (Mattiasson, B. and Holst, O., eds), pp. 173–188, Marcel Dekker
**26** Lye, G. J. (1997) *Biotechnol. Tech.* 11, 611–616
**27** Kulla, H. G. (1991) *Chimia* 45, 81–85
**28** Roddick, F. A. and Britz, M. L. (1997) *J. Chem. Technol. Biotechnol.* 69, 383–391
**29** van Der Wielen, L. A. M., Potters, J. J. M., Straathof, A. J. J. and Luyben, K. C. A. M. (1990) *Chem. Eng. Sci.* 45, 2397–2404
**30** Vicenzi, J. T., Zmijewski, M. J., Reinhard, M. R., Landen, B. E., Muth, W. L. and Marler, P. G. (1997) *Enzyme Microb. Technol.* 20, 494–499
**31** Chan, E. C. and Kuo, J. (1997) *Enzyme Microb. Technol.* 20, 585–589
**32** Schindler, J. (1982) *Ind. Eng. Chem., Prod. Res. Dev.* 21, 537–539
**33** Lynch, R. M., Woodley, J. M. and Lilly, M. D. (1997) *J. Biotechnol.* 58, 167–175
**34** Chauhan, R. P., Woodley, J. M. and Powell, L. W. (1997) *Biotechnol. Bioeng.* 56, 345–351
**35** Held, M., Schmid, A., Kohler, H-P. E., Suske, W., Witholt, B. and Wubbolts, M. G. (1999) *Biotechnol. Bioeng.* 62, 641–648
**36** Ribeiro, M. H. L., Prazeres, D. M. F., Cabral, J. M. S. and da Fonseca, M. M. R. (1995) *Bioprocess Eng.* 12, 95–102
**37** Kim, G-J. and Kim, H-S. (1994) *Biotechnol. Lett.* 16, 17–22
**38** Ramström, O., Ye, L., Krook, M. and Mosbach, K. (1998) *Chromatographia* 47, 465–469
**39** Paiva, A. L. and Malcata, F. X. (1997) *J. Mol. Catal. B Enzym.* 3, 99–109
**40** Chauhan, R. P., Woodley, J. M. and Powell, L. W. (1996) *Ann. New York Acad. Sci.* 799, 545–554
**41** Woodley, J. M., Mitra, R. K. and Lilly, M. D. (1996) *Ann. New York Acad. Sci.* 799, 434–445
**42** Brocklebank, S. P., Mitra, R. K., Woodley, J. M. and Lilly, M. D. (1996) *Ann. New York Acad. Sci.* 799, 729–736
**43** Lilly, M. D. and Woodley, J. M. (1996) *J. Ind. Microbiol.* 17, 24–29
**44** Wulff, G. (1995) *Angew. Chem., Int. Ed. Engl.* 34, 1812–1832
**45** Mosbach, K. and Ramström, O. (1996) *Biotechnology* 14, 163–170
**46** Whitcombe, M. J., Esther Rodriguez, M., Villar, P. and Vulfson, E. N. (1995) *J. Am. Chem. Soc.* 117, 7105–7111
**47** Sutherland, I. A. *et al.* (1988) *J. Liq. Chromatogr.* 21, 279–298
**48** Sutherland, I. A. (1987) *Lab. Pract.* 36, 37–42
**49** Bruce, L. J. and Daugulis, A. J. (1991) *Biotechnol. Prog.* 7, 116–124
**50** Bousquet, O. R., Braun, J. and Le Goffic, F. (1995) *Tetrahedron Lett.* 36, 8195–8196
**51** Ma, Y. and Ito, Y. (1995) *Anal. Chem.* 67, 3069–3074
**52** Arnold, F. H. (1996) *Chem. Eng. Sci.* 51, 5091–5102
**53** Crameri, A., Raillard, S-A., Bermudez, E. and Stemmer, W. P. C. (1998) *Nature* 391, 288–291

**Students**

Did you know that you are entitled to a 50% discount on a subscription to *TIBTECH*? See the subscription order card bound into this issue for more details.