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Chemical Engineering Science 61 (2006) 6646–6652

Chemical Engineering Science

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Process limitations in a whole-cell catalysed oxidation: Sensitivity analysis

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Received 20 December 2005; received in revised form 19 May 2006; accepted 4 June 2006
Available online 10 June 2006

Abstract

Biocatalytic oxidation processes have to date presented major problems for scale-up, in part due to the complexity of the number of process variables. In this paper we have analysed the key limitations in such processes using the Baeyer–Villiger monooxygenase catalysed synthesis of optically pure lactones as an illustrative example. Limitations in product concentration, catalyst longevity and reaction rate were quantified and their effect on previously defined process metrics identified. Of particular interest is the way these metrics change with catalyst concentration. Using this assessment, the sensitivity of the metrics to potential changes to process and catalyst were analysed. We believe such an analysis is of general use to guide development efforts for a given biocatalytic reaction.

Keywords: Biocatalysis; Optimisation; Mathematical modelling; Enzyme; Oxidation; Baeyer–Villiger monooxygenase

1. Introduction

While the use of enzymes and whole-cells as biocatalysts offer superb possibilities for novel and attractive chiral chemistry, such reactions are frequently limited in terms of productivity even after judicious screening and suitable expression. This is well illustrated in the case of one of the most useful classes of all biocatalysts—the oxygenases. These bioconversions are practically limited in terms of implementation by substrate and product inhibition, adequate supply of oxygen (required both for metabolism and reaction) and biocatalyst longevity.

However, work on addressing process options to overcome such limitations has typically been undertaken on a case-by-case basis (Chen et al., 2002; Blayer et al., 1996) to determine the effectiveness of techniques such as *in situ* product removal (Lye and Woodley, 1999), reactant feeding (Mitra et al., 1998) and two-liquid phase biocatalysis (van Sonsbeek et al., 1993; Lye and Woodley, 2001). This has not therefore addressed generic approaches to assessing and analysing reaction limitations and potential improvements for a given class of reaction. The challenge is two-fold at this point. First, there is a need to identify a given bottleneck and secondly to assess the potential benefit gained from alleviating this. In this paper we present a method to address this latter problem using a simple form of regime analysis (Bhole and Joshi, 2005; Link et al., 2005). For biological systems, often this initial identification of bioconversion (Wolff et al., 1999) or scale-up regimes in bioreactors (Sweere et al., 1987) has been achieved by analysis of characteristic times. An extension of this argument is to use catalyst concentration to identify particular regimes. For example, in a whole-cell process when oxygen supply is limiting (i.e. an oxygen-limited regime) an increased cell concentration will mean that the reaction rate decreases because of competition for oxygen from metabolism (Baldwin and Woodley, in press; Duetz et al., 2001). In order to illustrate the techniques of regime analysis applied in this way we present data on the Baeyer–Villiger oxidation, catalysed by a recombinant *Escherichia coli.*

2. Baeyer–Villiger monooxygenase

Baeyer–Villiger monooxygenases (BVMOs) are known to convert a wide variety of cyclic ketones (Roberts and Wan, 1988; Willetts, 1997; Mihovilovic et al., 2002) and sulphoxides (Chen et al., 1999; Colonna et al., 1998) into optically pure products and exist in a wide range of organisms including

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0009-2509/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ces.2006.06.007

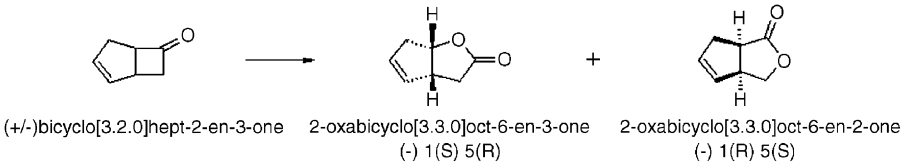

Fig. 1. Conversion of bicyclo[3.2.0]hept-2-en-6-one to (1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one and (1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one by cyclohexanone monooxygenase in *Escherichia coli* TOP10 pQR239.

Table 1
Improvements in process metrics for Baeyer–Villiger-mediated biocatalytic processes using whole-cell *E.coli* TOP10 pQR239 as catalyst

Process change	[Product] ($g_{prod}\,l^{-1}$)	$Yield_{product/catalyst}$ ($g_{prod}\,g_{dcw}^{-1}$)	Overall rate ($g_{prod}\,l^{-1}\,h^{-1}$)	Reference
Typical reaction	1.0	0.2	0.875	Extrapolated from (Doig et al., 2002)
Typical reaction with substrate feed	3.5	0.7	0.875	(Doig et al., 2002)
Optipore L-493 (ISPR)	19.1	1.1	0.423	(Simpson et al., 2001)
Recycle column + IS-SFPR	14.7	1.5	0.735	(Hilker et al., 2004a)
Bubble column + IS-SFPR	25.2	3.9	1.200	(Hilker et al., 2004b)
Typical reaction with 2× [oxygen] & low [catalyst]	3.5	3.5	0.648[a]	(Baldwin and Woodley, in press)

[a]Initial rate.

bacteria and fungi. In a series of publications we and others have reported the use of cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 (Alphand et al., 1998, 2003) for the conversion of bicyclo[3.2.0]hept-2-en-6-one to (1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one and (1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one (Fig. 1). However, not only does *A. calcoaceticus* contain a lactone hydrolase, reducing product yield, but moreover its use is precluded due to its status as a class 2 pathogen. As such, much work has focussed on the production of CHMO expressed in a recombinant *Saccharomyces cerevisiae* (Stewart et al., 1998) or *E. coli* (Doig et al., 2001; Mihovilovic et al., 2001). CHMO, as a type 1 BVMO, requires NADPH as a cofactor which is notoriously expensive, typically requiring recycle if used in an isolated enzyme system (Zambianchi et al., 2001; Kragl et al., 1996). Consequently such work by us and others using *E. coli TOP10* pQR239 (Doig et al., 2003) has commonly been carried out using a resting whole-cell catalyst format fed with a cosubstrate (glycerol) to effect *in vivo* NADPH recycle. *E.coli TOP10* pQR239 is capable of producing 500 U g^{-1} of CHMO (assayed as an isolated enzyme reaction on NADPH and the wild-type substrate cyclohexanone) from a batch fermentation. However, the maximum specific activity using a whole-cell catalyst on the substrate shown in Fig. 1 is approximately 55 U g_{dcw}^{-1} (Doig et al., 2002). A typical reaction (Fig. 1) using 5 $g_{dcw}\,l^{-1}$ whole-cell catalyst with substrate feeding and additional glycerol (for cofactor regeneration), produces 3.5 g l^{-1} of combined lactone with a yield ratio of 45:55 ((1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one to(1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one) from racemic ketone in 4 h (Doig et al., 2003). Furthermore, the two regioisomers are each optically pure (94% and 99% ee, respectively). While such purity is of great use to synthetic chemists, clearly such low productivity precludes scale-up to a commercial process and it is therefore instructive to examine the process limitations in a systematic manner. The three limits are defined beneath.

(a) *Substrate/product concentrations*: Scale-up work has determined that key to the process effectiveness is toxicity of the substrate and products towards the catalyst, with maximal activity being observed at ketone concentrations around 1.0 g l^{-1} and activity reduced to zero as combined lactone concentrations approach 4 g l^{-1} (Chen et al., 2002). This necessitates the use of productivity enhancing methods such as substrate feeding (Bird et al., 2002), *in situ* product removal (Simpson et al., 2001) or *in situ* substrate feeding and product recovery (Hilker et al., 2004a). The effect of implementing these process changes can be seen in Table 1.

(b) *Biocatalyst longevity*: Operational catalyst activity has been shown to decrease over time. Experiments carried out by us, have indicated that the whole-cell catalyst has a limited longevity of 450 min under typical reaction conditions (Doig et al., 2002). However, in experiments where aqueous concentrations of ketone and lactone have been kept below their inhibitory limits, such as those using *in situ* substrate feeding and product removal, catalyst has been shown to be still active up to 24 h after the start of the reaction (Hilker et al., 2004a). However, as the model we present here is based on aqueous reactions carried out in the absence of product recovery and at lower catalyst concentrations than those of Hilker and coworkers (Hilker et al., 2004a) we have assumed the intrinsic longevity of the biocatalyst to be the first figure of 450 min in the analysis that follows. Clearly in operation, stability is based on longevity as well as exposure to substrate and/or product concentrations. Furthermore, we recognise that taking a finite time point for

the longevity is a significant simplification—in reality it is a function of time. Nevertheless, the conceptual and mathematical gains in such a simplification justify the assumption made here.

(c) *Oxygen requirements*: Due to the stoichiometric requirement for oxygen, at high cell concentrations oxygen supply becomes limiting due to competition with metabolism (van Beilen et al., 2003). The oxygen requirement is a function of whole-cell catalyst concentration and careful choice of catalyst concentration for the particular reaction is required. Techniques to enhance oxygen mass transfer such as increasing pressure in the reaction vessel (Duetz et al., 2001), increasing surface area to volume ratio and residence time of the air bubble using a bubble column (Hilker et al., 2004a) or increasing the oxygen partial pressure (Baldwin and Woodley, in press) have all been used to assess this limitation.

Given the amount of information available, this poses a very useful basis to begin regime analysis, although the method we present, we believe to be quite general.

3. Regime identification

Analysis of past data, in the form of product concentration–time ([*P*]-*t*) profiles, can be used to identify a series of process limitations. This can be seen in Fig. 2, in which a given progress curve fits within the non-limited (un-shaded) area, the boundaries of which can be described by a series of three linear equations. Product concentration–time profiles that cross line A, will be limited by biocatalyst longevity and characterised by the biocatalyst longevity limit (BLL) which bounds the right of the un-shaded area, described by (1) below (where t is time in minutes and BLL is the time for which biocatalyst activity is above zero and determined to be 450 min (Chen et al., 2002)). Increasing biocatalyst concentration and thereby reaction rate, results in the dominating regime becoming limited by the product concentration limit (PCL, line B) which bounds the top of the un-shaded area and is characterised by (2) below (in which $[P]$ is product concentration in $g_{prod}\,l^{-1}$ and PCL is the concentration at which product becomes inhibitory also in $g_{prod}\,l^{-1}$ and set at $3.5\,g_{prod}\,l^{-1}$ (Chen et al., 2002)). However, rate cannot be increased infinitely by increasing catalyst concentration as there exists a maximum rate achievable in a given reactor system, in this case determined by the oxygen supply characteristics of that reactor, giving rise to the oxygen supply limit (OSL). The OSL (for convenience measured here in $g_{dcw}\,l^{-1}$) is the maximum catalyst concentration that can be provided to a particular reactor configuration at which the maximum specific rate still applies. OSL can be described as a function of product concentration as in (3) below, in which $r'_{p\,max}$ is the maximum specific rate ($g_{prod}\,g_{dcw}^{-1}\,h^{-1}$).

$$t = \mathrm{BLL}, \tag{1}$$

$$[P] = \mathrm{PCL}, \tag{2}$$

$$\mathrm{d}[P]/\mathrm{d}t = (r'_{p\,max} \times \mathrm{OSL})/60. \tag{3}$$

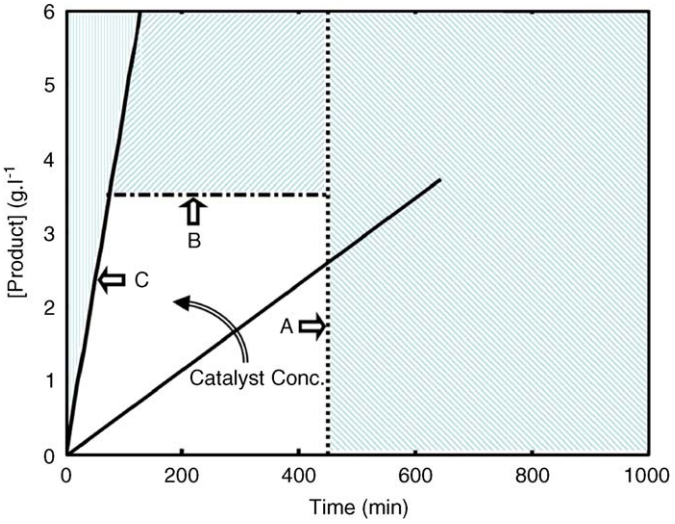

Fig. 2. [Product]–time profiles showing regimes: (C) oxygen and therefore rate limited regime, (B) [product] and therefore product inhibition-limited regime and (A) biocatalyst longevity-limited regime.

In a previous paper (Baldwin and Woodley, in press) we reported data from two 50 l reactions, run at catalyst concentrations of $5\,g_{dcw}\,l^{-1}$ and $10\,g_{dcw}\,l^{-1}$, both of which were limited by oxygen supply. In the same paper we reported a maximum specific rate for this strain of $0.65\,g_{prod}\,g_{dcw}^{-1}\,h^{-1}$ ($r'_{p\,max}$) when oxygen was not limiting. From these data it can be interpolated that a lower biocatalyst concentration will provide this maximum specific rate under the same reaction conditions and provide the OSL (Baldwin and Woodley, in press) and this was calculated to be $4.3\,g_{dcw}\,l^{-1}$ corresponding to an initial rate of $2.8\,g_{prod}\,l^{-1}\,h^{-1}$. This value of OSL can be seen in Fig. 2 as line C.

From this figure, it is clear that there are three main limitations to the productivity of a whole-cell CHMO-catalysed ketone oxidation, namely; rate limitations due to oxygen supply, product concentration limitations due to inhibition and catalyst limitations due to longevity. All three limitations will need to be balanced in a given case dependent upon process economics.

Key to the analysis is the choice of several process metrics (see Fig. 3), which adequately describe the effect of limiting regimes and simultaneously allow for sensitivity analyses of the varying reaction conditions:

(1) product concentration ($g_{prod}\,l^{-1}$),
(2) maximum initial reaction rate ($g_{prod}\,l^{-1}\,h^{-1}$) and
(3) biocatalyst efficiency ($g_{prod}\,g_{dcw}^{-1}$).

It is instructive to plot these metrics as a function of catalyst concentration (Fig. 4). Reaction rate is described by the maximum specific activity of $0.65\,g_{prod}\,g_{dcw}^{-1}\,h^{-1}$ in a non-limited regime (Baldwin and Woodley, in press), but becomes oxygen limited at $4.3\,g_{dcw}\,l^{-1}$ catalyst (regime C). Product concentration is dependent on biocatalyst stability at low biocatalyst concentrations (regime A), reaching the inhibitory limit of $3.5\,g_{prod}\,l^{-1}$ when catalyst concentration exceeds $0.7\,g_{dcw}\,l^{-1}$ (regime B). Finally, biocatalyst efficiency is plotted by the division of the product concentration by catalyst concentration.

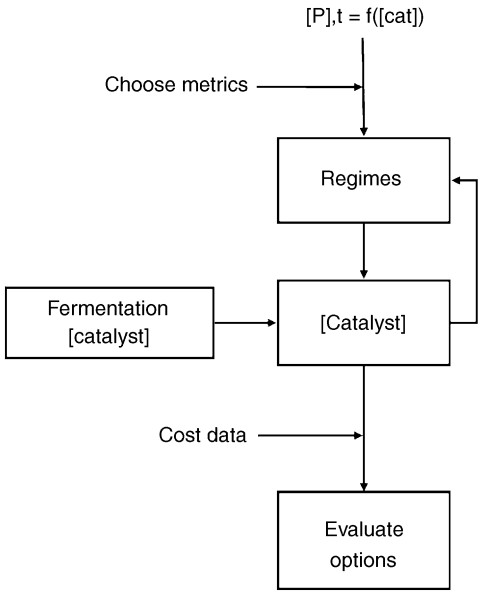

Fig. 3. Paradigm for regime analysis.

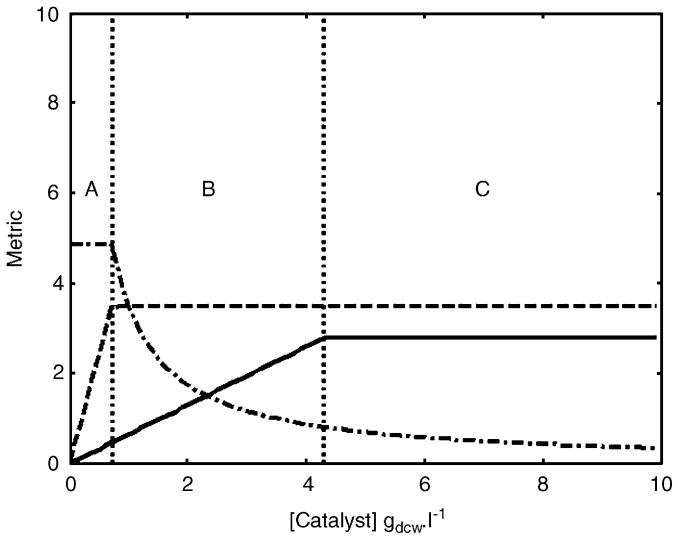

Fig. 4. Catalyst concentration, its associated limiting regimes and their effect on process metrics: (A) longevity-limited, (B) product-limited and (C) rate-limited regimes. (—), Rate $g_{prod}\,l^{-1}\,h^{-1}$; (- - -), product concentration $g_{prod}\,l^{-1}$; (-,-), biocatalyst efficiency $g_{prod}\,g_{dcw}^{-1}$; (. . .), regime limits.

4. Modelling

In order to carry out sensitivity analyses on these metric-catalyst concentration plots, a simple Matlab® model was created relying on four pieces of data key to the three limitations; maximum specific rate achievable for the system ($r'_{P\,max}$), the maximum time for which the enzyme is active (BLL), the highest biocatalyst concentration at which the reaction is unhindered by oxygen supply (OSL) and the maximum product concentration attainable without inhibition (PCL).

For metric-catalyst concentration plots, inequality driven linear equations were used to describe these relationships. For metric 1, product concentration ($[P]$, $g_{prod}\,l^{-1}$), is limited by the point at which catalyst concentration (X_{PCL}, $g_{dcw}\,l^{-1}$) and therefore rate, is so low the final product concentration is set by BLL rather than PCL. This is described in (4). Eqs. (5) and (6), describe the inequality.

$$X_{PCL} = PCL/(BLL/60) \cdot r'_{P\,max}, \tag{4}$$

$$X \leqslant X_{PCL} \quad \text{then } [P] = (PCL/X_{PCL}) \cdot X, \tag{5}$$

$$X \leqslant X_{PCL} \quad \text{then } [P] = PCL. \tag{6}$$

For metric 2, initial rate (r_p, $g_{prod}\,l^{-1}\,h^{-1}$) the equality was based on the OSL ($g_{dcw}\,l^{-1}$) as

$$X \leqslant OSL \quad \text{then } r_p = r'_{p_{max}} \cdot X, \tag{7}$$

$$X > OSL \quad \text{then } r_p = r'_{p_{max}} \cdot OSL. \tag{8}$$

Finally metric 3, biocatalyst efficiency, η_X, ($g_{prod}\,g_{dcw}^{-1}$) is described by

$$\eta_X = [P]/X. \tag{9}$$

5. Sensitivity analysis

Using the model, hypothetical changes to the limitations can be explored, by altering each of the initial data in turn and investigating the effects of that process change on each metric. Fig. 5, shows that increasing oxygen supply to the reaction and thereby OSL, increases rate to the maximum initial rate at any chosen catalyst concentration. Increasing PCL can be seen to increase final product concentration proportionally (Fig. 6) and as such PCL also affects biocatalyst efficiency. For example increases in PCL increase the range of catalyst concentrations over which biocatalyst efficiency is at a maximum, but in addition any change in PCL proportionally increases the biocatalyst efficiency at a given catalyst concentration (Fig. 7). In addition, an increase in PCL will also lead to a more stable biocatalyst. In order to simplify this rather complex issue we have used a value to BLL based on catalyst longevity alone

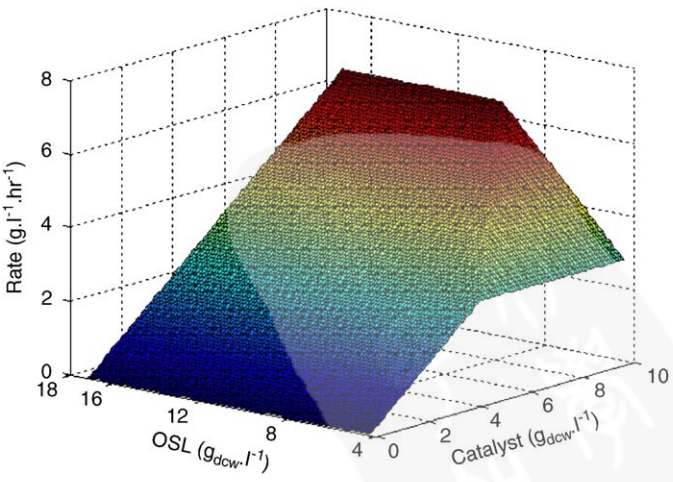

Fig. 5. Effect of change in OSL with catalyst concentration on initial rate.

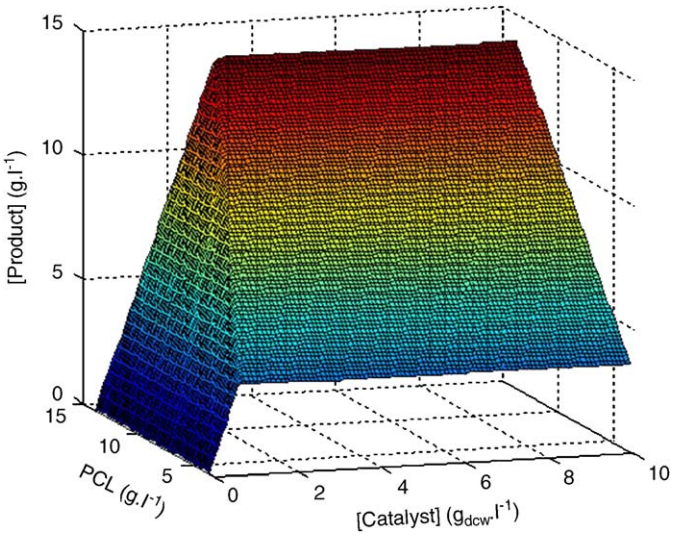

Fig. 6. Effect of change in PCL with catalyst concentration on final product concentration.

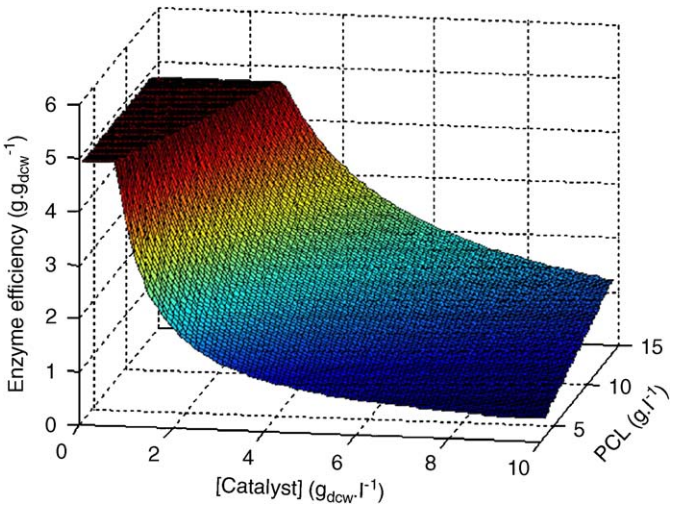

Fig. 7. Effect of change in PCL with catalyst concentration on biocatalyst efficiency.

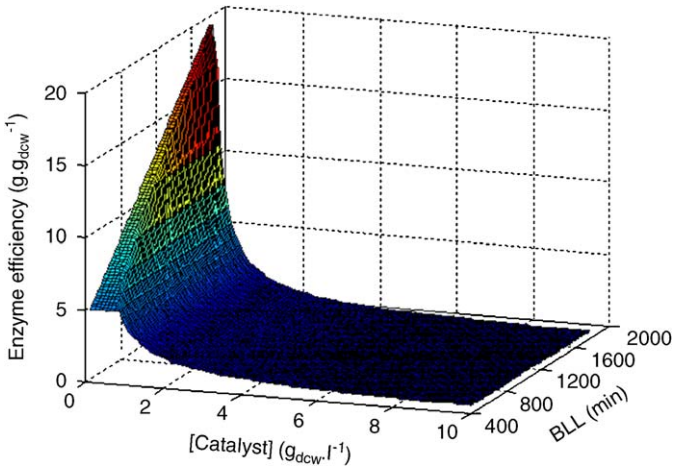

Fig. 8. Effect of change in BLL with catalyst concentration on biocatalyst efficiency.

Table 2
Sensitivity of process metrics to a two-fold reduction in process limitation at a catalyst concentration of $6\,g_{dcw}\,l^{-1}$

Increase in process metric	2 × PCL	2 × OSL	2 × BLL
Initial rate ($g\,l^{-1}\,h^{-1}$)	No change	1.4×	No change
Biocatalyst efficiency ($g\,g_{dcw}^{-1}$)	2×	No change	No change
Final product concentration ($g\,l^{-1}$)	2×	No change	No change

(i.e. independent of substrate and product concentration). Fig. 8 describes the effect of BLL on biocatalyst efficiency, where improvements can be seen to be restricted to low catalyst concentrations. This is because the reaction rate is sufficiently high (at reasonable catalyst concentrations) that the PCL is reached before the biocatalyst loses activity. The changes to process metrics are summarised in Table 2 for a catalyst concentration of $6\,g_{dcw}l^{-1}$ and a doubling in OSL, PCL and BLL.

It is apparent from these figures that the benefit of an individual process limitation change is dependent upon the prevailing regime and consequently catalyst concentration. For example, it can be seen that improving BLL is unnecessary unless the reaction is to be carried out at extremely low biocatalyst concentrations, but furthermore that at catalyst concentrations consistent with previous work, both biocatalyst efficiency and product concentration are greatly affected by improvements in the product limitation, PCL. As such it is clear that further process development be directed into improving the final product titre rather than improving catalyst longevity. Interestingly the methodology we propose here is applicable to the range of oxidative biocatalytic reactions, although such findings as the need to improve PCL (rather than BLL) are of course system specific. With regards to reaction rate, increases in rate and hence reductions in processing time could be achieved relatively easily by increasing oxygen supply to the catalyst, however this would require a cost–benefit analysis before such work proceeded due to the typical expense of such a step.

6. Catalyst concentration

Conventionally fermentation and biocatalytic conversion have been carried out in the same bioreactor. However, changing the bioreactor with the consequent option of intermediate dilution or concentration may have the added benefit of adjusting the catalyst concentration, as well as changing the medium (giving a cleaner stream for downstream processing). The extent to which this is feasible is shown schematically in Fig. 9 which shows the limits of the fermentation and dilution/concentration options. Where $[catalyst]_1$ and $[catalyst]_2$ are equal, a direct fermentation-reaction system is possible. To the left of this line lies a region in which dilution from the fermentation is required and to the right a region requiring concentration to make best use of the catalyst, with maximum catalyst concentration from the fermentation being limited by

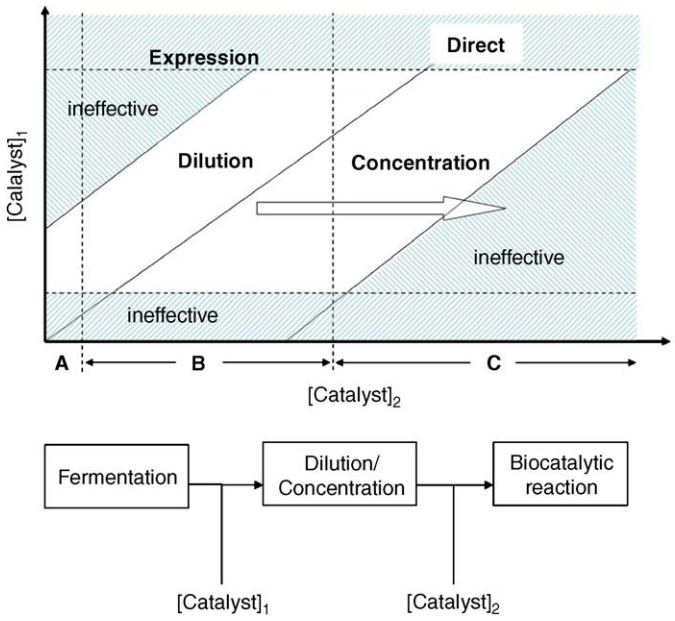

Fig. 9. Feasibility of dilution/concentration options between fermentation and reaction, showing regimes C (rate limited), B (product limited) and A (catalyst longevity).

expression levels. The shaded regions describe areas where the trade-off between fermentation catalyst concentration and reaction catalyst concentration is ineffective, which will be determined by the economics of a given process. Assuming cells are grown in high cell density fermentations then routinely processes will lie in the dilution portion of the plot. The extent to which dilution is required will be determined by the maximum cell concentration from the fermentation while maintaining expression. A quantitative version of this plot can be used to design the process together with information about the optimal cell concentration for use in the reactor (based on the previous sensitivity analysis, the regimes (A, B and C) can be superimposed on the plot).

7. Concluding remarks

The tools for analysis presented in this paper provide the basis for regime analysis to identify process limitations and sensitivity analyses to determine the impact of changing these bottlenecks. Together with cost–benefit analyses the process engineer is provided with a clear indication of where it is necessary to make changes to improve a process. Table 3 indicates potential techniques to afford such improvements. In this paper the productivity of the CHMO-catalysed whole-cell synthesis of an optically pure lactone has been shown to be very dependent upon biocatalyst concentration. There is an optimum concentration based on a given oxygen supply rate and this serves to limit the biocatalyst concentration in the reaction phase. One consequence of this is that the fermentation may not be suited to produce biomass at this optimal concentration. Hence, the separation of catalyst production from use is well justified. The work presented here has also identified three limiting factors and at suitable catalyst concentrations it is clear that work should focus first on supply of oxygen (or reduction in oxygen demand e.g. by alteration of metabolism) and secondly on effective methods of *in situ* product recovery. The improvement in catalyst stability is only useful at very dilute cell concentrations and not appropriate for scaleable systems. Finally, we have presented a tool using regime analysis to enable identification of targets for enhancement. The model has been used not for optimisation but to guide efforts to remove limiting bottlenecks. The tool would appear to be general and should prove a powerful method to guide development.

Table 3
Potential techniques to improve process limitations determined by regime analysis

Limitation	Potential solutions
Oxygen supply	Enriched air High pressure Agitation speed/aeration rate *Engineer cell to require less oxygen for metabolism*
Product removal	*In situ* product removal *Engineer enzyme/cell to be more product tolerant*
Biocatalyst longevity	Immobilise catalyst *Engineer enzyme/cell to be more stable*

Notation

BLL	biocatalyst longevity, min
OSL	limiting biocatalyst concentration based on oxygen supply, $g_{dcw}\,l^{-1}$
$[P]$	product concentration, $g\,l^{-1}$
PCL	inhibitory product concentration, $g\,l^{-1}$
r_P	initial product formation rate, $g\,l^{-1}\,h^{-1}$
r'_P	initial specific product formation rate, $g\,g_{dcw}^{-1}\,h^{-1}$
$r'_{P,max}$	maximum initial specific product formation rate, $g\,g_{dcw}^{-1}\,h^{-1}$
t	time, min
X	biocatalyst concentration, $g_{dcw}\,l^{-1}$
X_{PCL}	product limiting biocatalyst concentration, $g_{dcw}\,l^{-1}$

Greek letter

η_X	biocatalyst efficiency, $g\,g_{dcw}^{-1}$

Acknowledgements

The authors are grateful to the European Commission (QLK3-CT-2001-00403) for financial support of this work and to R. Wohlgemuth, R.E. Cripps, R. Furstoss, G. Carrea and A. Sayar for stimulating discussions.

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