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Determination of reactor operation for the microbial hydroxylation of toluene in a two-liquid phase process

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SUMMARY

Application of biotransformations to the synthesis of industrial chemicals is in part limited by a number of process challenges. We discuss the conversion of toxic, poorly water-soluble organic substrates by whole cells, using as an illustrative example the specific hydroxylation of toluene to toluene *cis*-glycol by *Pseudomonas putida* UV4. Toxic effects may be eliminated through the introduction of tetradecane, to partition toluene away from the biocatalyst, to give product concentrations of 30–60 g L^{-1}, in a two-liquid-phase reactor. The operational limits of this system have been experimentally determined and are presented in the form of windows of operation.

INTRODUCTION

Interest in the use of biocatalysts for the synthesis of industrial chemicals is growing in the light of environmental and regulatory trends, since biotransformations frequently operate at ambient temperature, near neutral pH and at ambient atmospheric pressure [6,24,25]. However the use of biological catalysis to carry out industrially useful chemistry is often difficult, involving the challenges of substrate and/or product toxicity or inhibition, high dilution and the use of pH- and temperature-labile biocatalysts. Biological and process solutions exist to many of these problems [1,6,8,10,23], although there are few established methods to compare strategies and techniques [20,30]. In this paper we discuss the use of biocatalysts to carry out conversions where the reactants are toxic to the biocatalyst, in order to exemplify the design approach that may be taken. Many industrially important biotransformations fall into this category [e.g. 18] and various methods have been cited to counter the problem; for example, the use of membrane reactors (separating the toxic bulk reactant from the biocatalyst), feed-back control systems [12] or via the introduction of a second phase to act as a reservoir for the toxic reactant [7,28]. In particular there has been interest in recent years in carrying out biotransformations (using either isolated or immobilised enzymes or whole cells) in the presence of a second, organic, phase. Many examples demonstrating the rationale for introducing an organic liquid phase can be found in the literature [19,22,26,27]. Here we discuss use of an organic phase (tetradecane) to partition a toxic reactant in the system. This has been examined using the specific hydroxylation of toluene to toluene *cis*-glycol (*cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene, TCG) by *Pseudomonas putida* UV4, based on the original work of Gibson [11] on oxidation of aromatics. Chemistry based on the use of such compounds has been studied extensively [5,16]. *Pseudomonas putida* UV4 is a blocked mutant capable of only the first stage in the metabolism of toluene (Fig. 1), preventing subsequent dehydrogenation to 3-methyl catechol. It is used as a whole cell biocatalyst, due to the need to supply stoichiometric amounts of NADH. This can be circumvented in the cell by the use of ethanol as a co-substrate and using the cell's mechanism for NADH regeneration. Additionally the three-protein, enzyme complex is unstable upon isolation [2]. The reaction requires molecular oxygen. The reactant, toluene, is toxic to the cell and poorly water-soluble while the product, TCG, is non-inhibitory and water-soluble [3].

REACTOR OPERATION WITH A SECOND, ORGANIC, PHASE

Characterisation of the hydroxylation of toluene by *Pseudomonas putida* UV4 has given accurate limits within which the

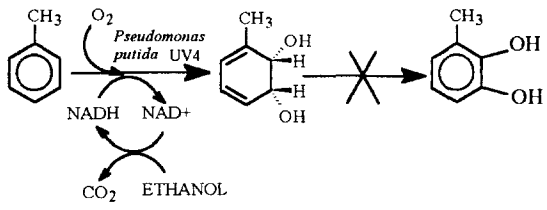

Fig. 1. Hydroxylation of toluene to TCG, by *Pseudomonas putida* UV4.

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aqueous phase toluene concentration should be controlled (10–40% of aqueous toluene saturation, or 0.54–2.4 mM) to avoid poor reaction rates (aqueous toluene concentrations <0.54 mM) and toxic effects (aqueous toluene concentrations >2.4 mM) [29]. The need to control the aqueous toluene concentration is imperative for effective reactor operation. However in a two-liquid-phase system, toluene is preferentially present in the organic solvent rather than the aqueous medium. Hence, the introduction of an organic solvent will partition toluene away from the aqueous phase (Fig. 2), where we have established that the biotransformation occurs [29]. Therefore, high concentrations of toluene are achievable in the reactor, in batch mode, while maintaining a low concentration within the aqueous phase. The aqueous phase toluene concentration is determined in part by the partition of toluene between the two phases and can be controlled by adjustment of the organic phase toluene concentration. The overall reactor toluene concentration is determined both by the organic phase toluene concentration and by the phase ratio (volume fraction of the reactor that is organic phase) [22].

PROCESS IMPLICATIONS OF INTRODUCING A SECOND, ORGANIC, PHASE

The introduction of an organic liquid phase forms a heterogeneous reaction medium, necessitating the reaction be performed in a stirred tank reactor, so that the phases are well mixed (or other configurations where high interfacial areas are developed e.g. membrane reactors). It has been cited that the liquid–liquid interface may be detrimental to the biocatalyst [4,14,15,21]. Additionally, where the biocatalyst comes into direct contact with the interface it may be exposed to high, toxic, levels of toluene. Use of a well-mixed heterogeneous

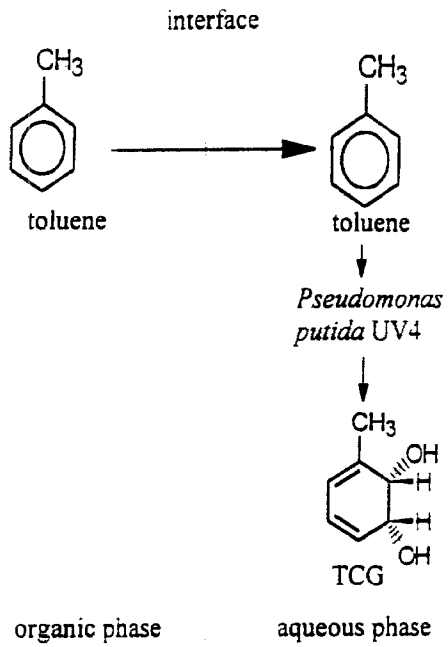

Fig. 2. Schematic illustration of toluene hydroxylation in a two-liquid-phase system. Toluene is preferentially present in the organic phase. No TCG was detected in this phase.

reaction medium may also lead to problems downstream by the formation of stable emulsions, as is well known [18]. This then requires complex downstream processing in order to recover the product entrapped in the emulsion, in this case from the aqueous phase. These issues limit the applicability of the two-liquid-phase method. Therefore we have focused experimental work on biocatalyst operational stability, in particular liquid–liquid interfacial inactivation, and emulsification in order to further understand the limitations and application of this method in reducing the toxic effects of poorly water-soluble organic substrates.

MATERIALS AND METHODS

Biocatalyst production

Pseudomonas putida UV4 was kindly supplied by Zeneca BioProducts (formerly ICI BioProducts and Fine Chemicals, Billingham, Cleveland, UK) and was stored on nutrient agar plates. Active colonies (blue) from indole agar plates (nutrient agar containing 0.1 g L^{-1} indole [17]) were used to inoculate 2 × 250-ml shake flasks containing carbon-limited media [9]. The biocatalyst was grown in a 2-litre working volume fermenter (MBR Bioreactor, B Braun Biotech, Aylesbury, Bucks, UK). The fermentation medium was the same as for the shake flasks. pH was maintained at 7.0 by the addition of 3 M KOH. Glucose (125 g L^{-1}) was fed continuously to the reactor (22.7 ml h^{-1}) for 24 h (agitation 800 r.p.m., air flow rate 4 L min^{-1} and temperature 28 °C). A final bacterial concentration of 9–10 g L^{-1} (dry weight basis) was achieved routinely.

Biocatalyst harvest

Cells were harvested in a 2-L Hi Spin Centrifuge (MSE Scientific Instruments, Manor Royal, Middx, UK), for 20 min at 6000 r.p.m. (4 °C). The biomass was immediately resuspended in 50 mM phosphate buffer, pH 7.5, for use in the biotransformation.

Two-liquid-phase biotransformation

Biotransformations were carried out in two identical reactors (MBR Bioreactor), with a working volume of 1.7 L at 28 °C, with an agitation speed of 800 r.p.m. and air flowrate of 4 L min^{-1}. The aqueous phase (containing biocatalyst at 4–6 g (dry weight) L^{-1}) at pH 7.5, was added to the reactor with 15 ml of ethanol, as co-substrate. This was left for approximately 30 min, under reaction conditions or, until the DOT stabilised (normally around 80%). The organic phase was then added to the reactor to give an initial phase ratio (volume fraction organic phase) of 0.3 (510 ml organic, 1190 ml aqueous). The organic phase was composed of 20% (v/v) toluene in tetradecane to give a reactor toluene concentration of 0.55 M. These values were used unless otherwise stated.

Biocatalyst activity (test flask activity assay)

This assay was based on the hydroxylation of toluene to TCG, supplying toluene from a vapour phase. The assay was carried out in a 250-ml conical flask, with centre well and a

foam bung. Phosphate buffer (9 ml, 50 mM, pH 7.5) was placed in the outer well of the conical flask. Toluene (0.5 ml) was added to the inner well and the flask incubated for 1 h in an orbital shaker at 250 r.p.m. (28 °C). Cell suspension (1 ml) was added to the outer well (with an OD 670 < 1.0) of the pre-incubated flask. After initial samples were taken, ethanol (40 μl) was added to the outer well of the flask. Samples (1 ml) were taken at intervals and ODs measured at 670 nm and 265 nm (blanked appropriately for nucleic acid release) in order to determine dry weights and TCG concentrations respectively. Hence specific activities were determined (specific activity (g g^{-1} h^{-1}) = TCG (g L^{-1})/(cell dry weight (g L^{-1}) × time (h))).

Emulsion characterisation

Emulsions were characterised by their ability to separate into their constituent phases after centrifugation. The reaction mixture (40 ml) was put into a 50-ml polypropylene centrifuge tube and centrifuged for 20 min at 8850 × *g* (20 °C). The resulting phase volumes were measured and the percentage recovery of each phase determined.

HPLC analysis for TCG

TCG analysis was carried out using reverse phase HPLC (Thermo Separation Products, Stone, Staffs, UK), with automatic sample injection and a non-polar C18 column packed with Spherisorb S5OD52 (125 × 4.9 mm). The mobile phase (water, HPLC grade, methanol, sodium acetate trihydrate and acetic acid, in the ratio 80:20:0.2:0.1) flowrate was 1 ml min^{-1} and TCG was detected at 270 nm after a retention time of 2.8 min. Calibration of the system was done before each use, using TCG provided by Zeneca BioProducts.

RESULTS

Reactor operation—biocatalyst operational stability

Figure 3 shows the results from a biotransformation (phase ratio 0.3 with 10% (v/v) toluene). TCG concentrations of 25 g L^{-1} were achieved which is a marked improvement on aqueous phase and fed-batch operations [21]. However even in a two-liquid-phase system maintaining sub-toxic concentrations of toluene in the aqueous phase, a decrease in residual dioxygenase activity was observed. The relation between productivity and biocatalyst stability has focused research on understanding the mechanisms of biocatalyst inactivation. Methods for improving stability have been examined by studying the effects of the reactor operational parameters upon residual dioxygenase activity.

Under exposure to biotransformation conditions (but with neither co-substrate nor second phase present), *Pseudomonas putida* UV4 showed a 50% loss in dioxygenase activity after 24 h (Table 1). However with the addition of ethanol (initial concentration 4 g L^{-1}) the cells retain dioxygenase activity over the same period. This indicates that potentially the biocatalyst is metabolising the co-substrate. Likewise for cells exposed to tetradecane (at phase ratios of 0.3 and 0.5) in the presence of ethanol there was no loss of biocatalyst activity over the 24-h period. This suggests that the presence of the

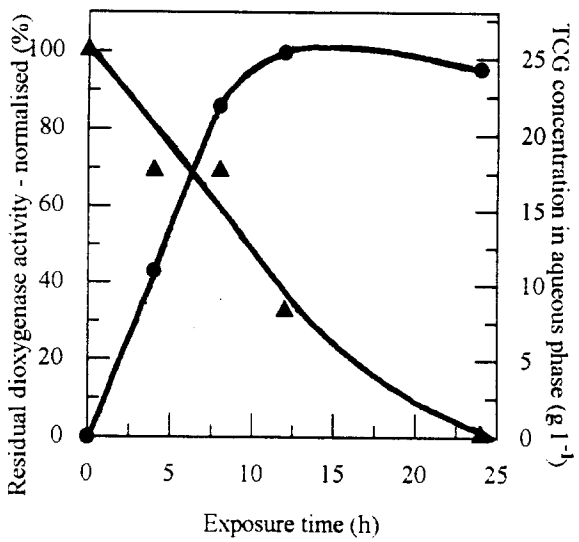

Fig. 3. Residual dioxygenase activity (▲) of *Pseudomonas putida* UV4 exposed in a two-liquid-phase system (phase ratio 0.3). The organic phase was tetradecane containing 10% (v/v) toluene. Resultant TCG in the aqueous phase (●). (100% activity ≡ 1.2 g g^{-1} h^{-1}).

tetradecane phase and hence the liquid–liquid interface has no detrimental effect on the residual dioxygenase activity. However, when toluene was added to the organic phase, at 20% (v/v), virtually all activity was lost in the first 4 h (Fig. 4). An organic phase toluene concentration of 20% (v/v) (1.83 M) will give an aqueous phase toluene concentration of 1.92 mM at equilibrium. The partition of toluene between tetradecane and buffer was previously established as thermodynamically ideal with a distribution coefficient of 981 [12]. Since an aqueous phase toluene concentration of less than 2.4 mM is non-toxic [29], introducing the organic phase at 20% (v/v) toluene in tetradecane should maintain sub-toxic levels of toluene in the aqueous phase. Hence the observed loss of activity reported in Fig. 4 was attributed to toluene exposure via the interface rather than the aqueous phase. When the experiment was repeated, this time with only 10% (v/v) toluene in the tetradecane (aqueous phase toluene concentration of 0.81 mM at equilibrium), 30% of the residual dioxygenase activity was still observed after 12 h, confirming this hypothesis (Fig. 4).

Catalyst supply—integration into the process

The results shown in Fig. 4 and Table 1 indicate the way in which reactor conditions may affect biocatalyst operational stability. Likewise, supply of the catalyst to the reaction may affect stability within the reactor. In standard experiments cells were harvested from the fermentation by centrifugation prior to resuspension. Figure 5 shows the residual dioxygenase activity of cells where the biotransformation immediately proceeded the fermentation, by direct addition of the organic phase to the fermentation broth. This eliminated the need for a harvesting stage or any storage of the biocatalyst. The biocatalysts that had not undergone a harvesting stage had a greater tolerance to the biotransformation conditions. The amounts of both aqueous and organic phase recovered were also slightly greater than those for the harvested biocatalyst.

TABLE 1

Effect of ethanol and tetradecane exposure on residual dioxygenase activity of *Pseudomonas putida* UV4

Exposure conditions		Residual dioxygenase activity[a] (hours)				
Ethanol	Tetradecane	0[b]	4[b]	8[b]	12[b]	24[b]
–	–	100	94	100	80	52
4 g L^{-1}	–	100	100	100	100	95
4 g L^{-1}	0.3[c]	100	91	90	100	100
4 g L^{-1}	0.5[c]	100	89	100	100	100

[a] % of control (100% activity ≡ 1.0 g g^{-1} h^{-1}).
[b] Exposure time.
[c] Volume fraction organic.

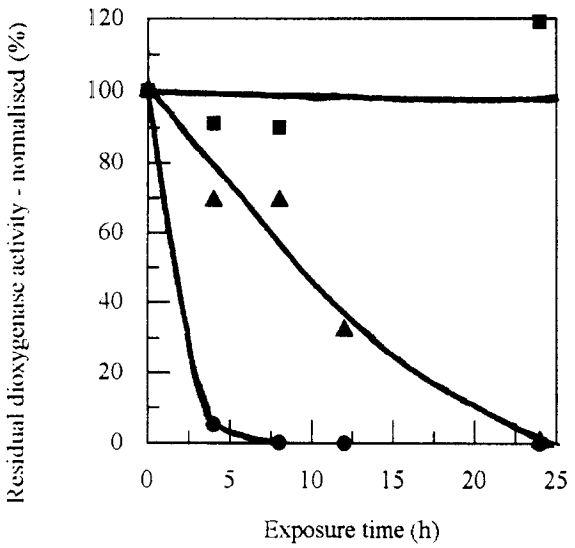

Fig. 4. Residual dioxygenase activity of *Pseudomonas putida* UV4 exposed in a two-liquid-phase system (phase ratio 0.3). The organic phase was tetradecane contaning 0 (■), 10 (▲) or 20 (●)% (v/v) toluene. (100% activity ≡ 0.77 g g^{-1} h^{-1}).

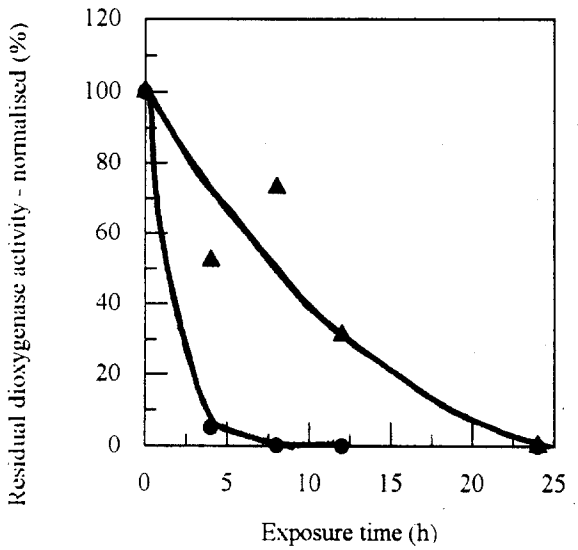

Fig. 5. Residual dioxygenase activity of *Pseudomonas putida* UV4 exposed in a two-liquid-phase system (phase ratio 0.3). The organic phase was tetradecane containing 20% (v/v) toluene. Biocatalyst having undergone a harvest and resuspension operation (●) and those used immediately after fermentation (▲). (100% activity ≡ 0.76 g g^{-1} h^{-1}).

This increase in tolerance of the non-harvested cells to the operational conditions could have been due to adverse effects of centrifugation and/or resuspension whilst harvesting. Alternatively, leaving the cells in the fermentation broth may have allowed the cells to metabolise further and alter their morphology giving them additional operational stability. Hence two unit operations may be lost giving an increased stability to the biocatalyst. However, biocatalyst concentrations will be relatively lower than those following a concentration step and it would be potentially difficult to extract the product from the complex fermentation medium rather than from buffer.

Pseudomonas putida UV4 is an unstable microorganism [12] and appears to lose dioxygenase acitivity rapidly after fermentation. The turn-around between fermentation and biotransformation has therefore always been kept to a minimum, never more than 3 h. Earlier work examined the storage of the biocatalyst, either as a cell pellet or resuspended in phosphate buffer [13]. Neither method proved reliable. When storage was required the biocatalysts were treated in the usual manner, until just before addition of the second, organic phase. They were left overnight (i.e. in a resuspended state in the presence of 4 g L^{-1} of ethanol) and the organic phase and necessary additional ethanol were added the next day. This method of storage minimised the loss of activity. Subsequent biotransformations showed no change in tolerance of the biocatalyst after storage (Fig. 6). However cells stored in ethanol showed no sign of subsequent emulsion formation at a phase ratio of 0.6, unlike cells stored without ethanol. We presume that cells stored in the presence of ethanol metabolise the ethanol (supported by the observation that ethanol was consumed overnight) and that this confers some maintenance of dioxygenase activity. It is likely the components of cell degradation are responsible for enhanced emulsion formation.

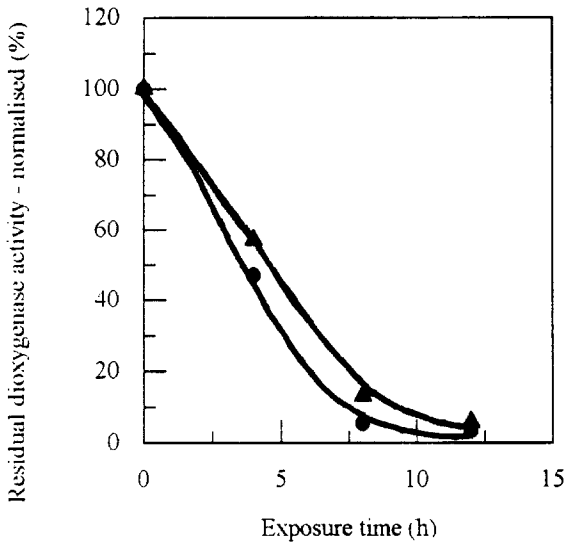

Fig. 6. Residual dioxygenase activity of *Pseudomonas putida* UV4 exposed in a two-liquid-phase system (phase ratio 0.3). The organic phase was tetradecane containing 20% (v/v) toluene. Biocatalyst having undergone overnight storage in the absence (●) and in the presence (▲) of 4 g L^{-1} ethanol. (100% activity ≡ 0.66 g g^{-1} h^{-1}).

Emulsification

The formation of large liquid–liquid interfacial areas and hence enhanced mass transfer between the phases may also create stable dispersions (emulsions). These emulsions may create problems for downstream processing, product recovery and solvent recycle. A simple centrifugation step recovered 80–98% of the aqueous phase from the reactor operations described above. Organic phase recovery was not as successful, recovering between 3 and 60%. Since the TCG product is predominantly aqueous phase-soluble (none was detected in the tetradecane phase), these results appear favourable. Nevertheless, the need to recover and recycle solvent is critical to environmental and process requirements.

Each of the systems described so far was run with the aqueous phase continuous (i.e. phase ratio <0.6). The logic for this was that the reaction occurred in the aqueous phase, with its requirements for pH control and DOT measurement. Further experimentation has revealed that running the system inverted (i.e. organic phase continuous at phase ratio >0.6) can lead to little or no emulsion formation (Table 2). A compromise between the benefit of this to downstream processing and the penalty of not being able to monitor the aqueous phase and the increased specific liquid–liquid interface that the biocatalyst is exposed to will have to be determined.

DISCUSSION

Operating windows

Operating windows can be used to illustrate schematically the effects of phase ratio, organic phase toluene concentration and biocatalyst concentration found in this paper. Figure 7 shows the effects of phase ratio and organic phase toluene concentration on aqueous phase kinetics. Between 8.5–25% (v/v) of toluene in tetradecane, the reaction in the aqueous

TABLE 2

Effect of phase ratio and biomass concentration on emulsion formation, after agitation[a] for 20 min. The phases either separated[b] immediately (+), formed a heavy emulsion (−) (no organic phase visible), or a light emulsion (+/−) where the phases separated but a small emulsion was visible between the phases

Phase ratio[c]	Biomass (g L^{-1})[d]			
	0	5	10	15
0	+	+	+	+
0.2	+	−	−	−
0.4	+	−	−	−
0.5	+	+/−	+/−	+/−
0.6	+	+	+	+/−
0.8	+	+	+	+
1.0	+	+	+	+

[a] 40-ml samples in a 50-ml polypropylene tube, agitation was by an overhead rushton turbine (24-mm diameter).
[b] Non-assisted separation (observation of separation).
[c] Volume fraction organic.
[d] Dry weight basis.

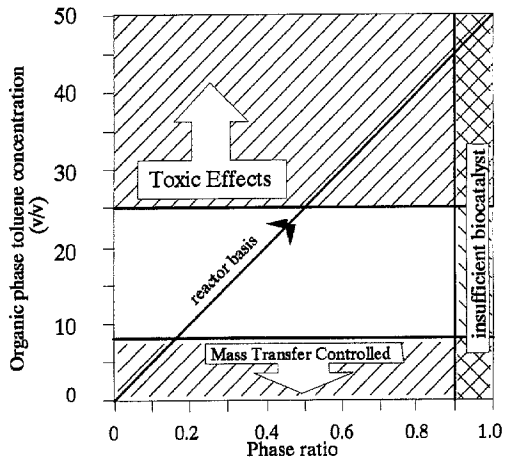

Fig. 7. Operating window: organic phase toluene concentration as a function of phase ratio (volume fraction organic).

phase is in the zero order kinetic regime (i.e. the hydroxylation rate is independent of toluene concentration), below this concentration the reaction is first order with respect to toluene. Ideally the reaction should be maintained within the zero order kinetic regime. At organic phase concentrations of toluene greater than 25% (v/v), the corresponding aqueous concentration is in the toxic regime. We have shown that the organic phase toluene concentration is critical and at sub-toxic levels of toluene in the aqueous phase there is still biocatalyst inactivation via the interface. Figure 8 shows the effects of phase ratio and aqueous phase biocatalyst concentration on emulsion formation. Emulsion formation can be avoided by operating with the organic phase continuous. However, the bulk reaction occurs in the aqueous phase, where the biocatalyst is present and hence there must be sufficient aqueous phase volume. In

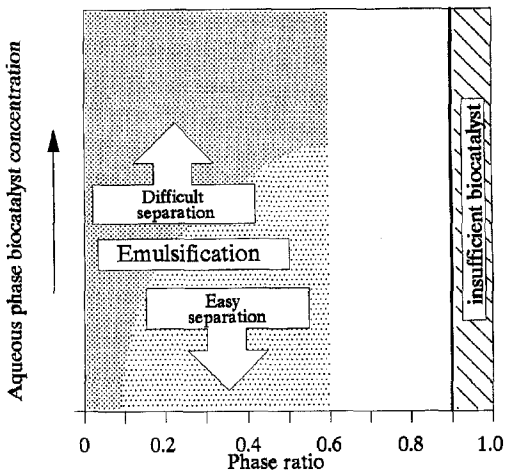

Fig. 8. Operating window: aqueous phase biocatalyst concentration as a function of phase ratio (volume fraction organic).

addition, emulsion formation is a function of the biocatalyst concentration in the aqueous phase. Reactions using higher concentrations of biocatalyst proved more difficult to separate. The clear areas in Figs 7 and 8 mark out possible operating areas or windows where operation should focus. The bounds to the areas show the limits to the productivity of the process and may direct future process research [32]. There is now a need to determine absolute values for the various parameters in the test system, so that the operating windows can be more precisely defined.

We have discussed how some of the parameters that affect a two-liquid-phase biotransformation (co-substrate addition, second phase and reactant concentration) may alter the biocatalyst stability and emulsion formation ability. There is no single optimal set of parameter values to afford the best biotransformation, but the illustrated windows may be used for process design. The two main problems of the process have been identified as biocatalyst stability and emulsion formation. A further feature of this process is the significance of the interfacial toluene concentration for biocatalyst stability. Further work should be aimed at determining whether this biocatalyst inactivation is a function of the toluene concentration at the interface, a function of the frequency of cell-interface contact or residence time, or a function of each of these [31]. Emulsion formation is an intrinsic problem associated with two-liquid-phase biotransformations but, we have shown that by careful control of the operating conditions this problem can be eased or even eliminated. The ability to overcome and understand some of these process problems, will direct work towards the goal of continuous operation of the system. Further research in this area will be described in detail in future publications.

Operating windows have been used as an aid to determine the interacting effects of phase ratio, biocatalyst concentration, and organic phase toluene concentration. This technique is applicable to many operations and is a powerful method for identifying further process research [32].

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