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A structured approach to design and operation of biotransformation processes

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A structured approach to design and operation of biotransformation (bioconversion) processes, based on previous case studies, has been developed. This requires knowledge of the key characteristics of a biotransformation which determine the constraints on process selection. The approach is illustrated for five biotransformations, two enzymic and three microbial. Some generic problems such as low water solubility and volatility of reactants, reactant and product toxicity have been identified. The microbial oxidations of toluene and fluorobenzene to toluene *cis*-glycol and fluorocatechol respectively by *Pseudomonas putida* have been used to illustrate how these constraints may be overcome by addition of tetradecane as a second liquid phase, use of a membrane oxygenator and introduction of *in situ* product removal.

Keywords: biotransformation; aromatic oxidation; process selection; *Pseudomonas putida*

Introduction

Many companies in the pharmaceutical, chemical and food sectors are involved in or interested in the development of biotransformations (bioconversions) exploiting the selectivity of enzymes. A wide range of biotransformations using free or immobilised enzymes or microorganisms is now operated in industry [6, 17]. However, nearly all of these have been designed on a case-by-case basis since few companies have experience in developing more than one or two such processes.

At University College London over the last 30 years we have had the opportunity to investigate over 20 biotransformations, in some cases working with companies on their commercial implementation. Some examples are listed in Table 1 (adapted from [17]), which indicates that, where appropriate, we have examined not only the biotransformation itself but also the production of the catalyst by fermentation, its subsequent isolation and immobilisation. Recently emphasis has also been placed on integration of product recovery from the biotransformation with the reaction itself [5, 9, 10, 22, 23]. This long-term research programme has allowed us to examine different classes of reactions and in some cases different approaches to the same reaction, eg ester hydrolysis by isolated enzyme, immobilised enzyme or intact microorganism [25, 26]. We have also identified common themes and problems to which we have sought solutions.

At the same time we have developed a structured approach to the design and operation of biotransformation processes [28]. The objective is rapid process selection to minimise the time and effort required to identify the most suitable process options and to develop a cost-effective, reliable and safe process. This involves an integrated approach to the selection and production of the biocatalyst, reactor design and operation, and product recovery (Figure 1).

The first step is the determination of the key characteristics of the biotransformation [28]. This requires information, which can be obtained either from published literature or by specifically designed experimental work, on: (i) the properties of the substrate(s) and product(s) (eg water solubility); (ii) the reaction (eg a pH change); (iii) the biocatalyst (eg cofactor requirement); and (iv) its interactions with the substrate(s) and product(s) (eg reaction kinetics). This knowledge makes it possible to identify constraints imposed by the reaction medium required, the reaction kinetics and the properties of the various forms of biocatalyst. This will eliminate many options, allowing further evaluation to concentrate on a small number of process designs. It is important to keep the amount of experimental effort to a minimum during this initial elimination stage, but more information on the biotransformation characteristics may be required later to allow further quantitative evaluation of the process options.

This approach is illustrated in Table 2, which summarises the constraints which were identified in this way during studies on five different biotransformations. Two involve the use of isolated or immobilised enzymes, an aldolase and transketolase, for carbon–carbon bond synthesis. The first catalyses a reversible reaction, the conversion of pyruvate and *N*-acetyl-D-mannosamine into *N*-acetyl-D-neuraminic acid [8, 15, 16], a valuable precursor of sialic acid derivatives [1]. The second reaction, the transfer of a two-carbon donor by transketolase, is also normally reversible but this constraint can be overcome by using hydroxypyruvate as the ketol donor in which case the second product is carbon dioxide and the reaction becomes irreversible [11]. This elimination of one constraint introduces two other constraints, gas production and a net reduction in pH during the reaction requiring addition of acid to maintain a constant pH value. The implications of these constraints for these reactions involving water-soluble substrates and

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Table 1

BIOTRANSFORMATION RESEARCH AT UCL

● WORK COMPLETED
○ WORK IN PROGRESS
□ FUTURE WORK

Enzyme	FERMENTATION	ISOLATION	IMMOBILISATION	BIOTRANSFORMATION
β-galactosidase	●	●	●	–
Penicillin acylase	●	●	●	●
Cholesterol oxidase	●	●	–	●
Chloroperoxidase	●	●	–	–
Lipase	–	–	●	●
Steroid dehydrogenase	●	–	●	●
Toluene dioxygenase	●	–	–	●
Halidohydrolase	●	●	●	●
Transketolase	○	○	○	○
Baeyer-Villiger oxygenase	○	○	□	□

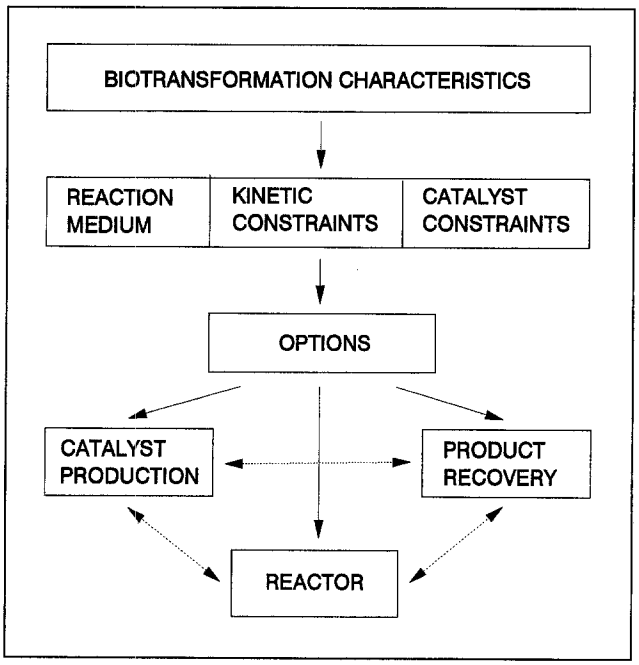

Figure 1 The main stages in biotransformation process selection.

products have been reported and discussed elsewhere [3, 29]. The constraints identified for three biotransformations involving the use of intact microorganisms are also listed in Table 2. In each case the biotransformation requires recycling of a cofactor through a second enzymic step. This requirement favours the use of the whole microorganisms rather than the isolated enzymes, especially as these are relatively unstable. The recombinant *Saccharomyces cerevisiae* containing a cloned P450 monooxygenase can be used for various biotransformations and the constraints listed in Table 2 are for oxidation of acetamidophenol [24], which is partially soluble in water (15 mM). The other two reactions involve poorly water-soluble aromatic substrates, toluene and fluorobenzene, to give toluene *cis*-glycol and fluorocatechol respectively, and will be discussed further in this paper.

Methods

Toluene hydroxylation by Pseudomonas putida UV4

Pseudomonas putida UV4, a mutant defective in benzene glycol dehydrogenase, was maintained on nutrient agar. Indole (0.1 g L^{-1}) was added to agar plates to check for reversion of colonies.

Fermentations were carried out at 28°C in a 2.5-L MBR fermentor (Alfa-Laval Engineering Ltd, Brentford, Middlesex, UK) with a working volume of 2 L and fitted with two six-bladed turbine impellers. The fermentation medium (1.46 L) containing (g L^{-1}): $NaH_2PO_4 \cdot 2H_2O$, 1.56; NH_4Cl, 4.5; KCl, 0.75; $Na_2SO_4 \cdot 10H_2O$, 0.66; citric acid, 0.42; $CaCl_2$, 0.002; Na_2MoO_4, 0.00004; and trace elements solution (5 ml L^{-1}). It was sterilised *in situ* at 121°C for 20 min. The vessel was inoculated with 50 ml of a shaken flask culture grown on an identical medium to which a solution containing 1 g glucose had been added. A glucose solution (125 g L^{-1}) was sterilised separately and fed to the fermentor at 1 g glucose h^{-1}, normally for 18 h. The pH

Table 2 Constraints on biotransformation

Characteristic	Isolated aldolase	Isolated transketolase	*S. cerevisiae* monooxygenase	*P. putida* dioxygenase	*P. putida* dioxygenase/ dehydrogenase
Reactant/product					
Melting/boiling pts				■ (S)	■ (S)
Aqueous solubility			□ (S,P)	■ (S)	■ (S)
Temperature stability		■ (S,P)			
pH stability		■ (S,P)		■ (P)	■ (P)
Reaction					
Equilibrium	■	■*			
pH shift		or ■*			
Gas consumption/production		and ■*	■	■	■
Interactions with catalyst					
Activity (pH, temperature)	■	■			
Stability (pH, temperature)					
Substrate inhibition	■				■
Product inhibition		■	■		
Substrate toxicity		■		■	
Product toxicity					■

■ = Constraint; □ = partial constraint; S = substrate; P = product.
*Constraints mutually exclusive depending on ketol donor.

was controlled at 7.0. The stirrer speed was increased from 700 to 900 rpm and the air flow rate from 30 to 50 L h^{-1} during the fermentation but the DOT was not controlled.

Biotransformations (1.7 L total volume) were done in aerated, agitated reactors containing 1.19 L of an aqueous suspension of bacteria (10–11 g dry wt L^{-1}) and 0.51 L of organic phase (phase ratio 0.3), which consisted of a 20% (v/v) solution of toluene in tetradecane.

Toluene *cis*-glycol in aqueous samples was assayed by reverse phase HPLC [7].

Conversion of fluorobenzene to fluorocatechol by Pseudomonas putida ML2

Pseudomonas putida ML2 was maintained on nutrient agar plates and sub-cultured weekly. The bacteria were produced in a 2.5-L stirred tank fermentor (MBR) maintained at 30°C and at pH 6.8 by the addition of either 1.5 M NH_4OH or H_2SO_4. The medium contained (g L^{-1}): fructose, 25; KH_2PO_4, 3; Na_2HPO_4, 3; $(NH_4)_2SO_4$, 1; $FeCl_2$, 0.016, and $CaCl_2$, 0.015; and trace elements solution (2 ml L^{-1}). The medium, without fructose which was sterilised separately, was sterilised at 121°C for 20 min, 1.5 L of medium containing fructose was inoculated with 100 ml of seed culture. This had been grown for 24 h in a 500-ml flask containing 100 ml of the salts medium to which 100 μl of benzene had been added and then sealed with a rubber seal.

Cultures were harvested at the end of the growth phase in an MSE 2-L Hi Spin centrifuge (MSE Ltd, Loughborough, Leics, UK) operated at 4000 × *g* and 4°C for 20 min. The bacteria were resuspended in 2 L of 100 mM phosphate buffer, pH 6.8, and normally used immediately in the biotransformation.

The resuspended bacteria were returned to the 2-L vessel and the reaction was started by switching on a pump feeding a 1:1 (v v^{-1}) mixture of ethanol and fluorobenzene.

For *in situ* product removal the bacterial suspension from the stirred tank was pumped through a packed bed (vol 0.35 L) of Norit pK13 (Norit Ltd, Glasgow, Scotland) at 25.8 L h^{-1}.

Fluorocatechol was extracted from the Norit pK13 by washing it with deionised water and then adjusting the pH of the suspension to 2.5 using 2.5 M H_2SO_4. The aqueous suspension was mixed with butyl acetate for 4 h after which the organic phase was separated and assayed for fluorocatechol.

A second circulation loop contained a membrane oxygenator through which the bacterial suspension was pumped at 4.8 L min^{-1}. The oxygenator consisted of a glass tube (length 1.4 m, diameter 0.012 m) housing 200 sealed dead-end hollow gas-permeable fibres (length 1.2 m, diameter 220 μm) (Membran Corp, Minneapolis, USA) pressurised by oxygen gas. The downstream ends of the fibres were free to move in the passing suspension. The DOT in the circulating bacterial suspension was controlled by regulating the oxygen pressure inside the fibres.

Fluorobenzene and fluorocatechol concentrations were determined using a Varian 3700 gas chromatograph (Varian Ltd, Walton-on-Thames, Surrey, UK) fitted with a 25-m capillary, non-polar, column (25C3/BPX5) and using helium as carrier gas. Injection of samples (1 μl) was done with a Carlo Erba autosampler (Thermo Separation Products Ltd, Stone, Staffs, UK) (model A200S). An automated programme raised the temperature linearly from 122 to 132°C. The injector and flame ionisation detector temperatures were 270° and 300°C.

Results

Toluene hydroxylation by Pseudomonas putida UV4

This reaction (Figure 2) using a mutant lacking an active dehydrogenase converts toluene to toluene *cis*-glycol (*cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene), a useful precursor for chemical synthesis of polyphenylenes [2] and other compounds of interest [13]. The reaction is without chemical precedent. Apart from the low aqueous solubilities of aromatic substrates such as toluene, other constraints have been identified, in particular substrate toxicity and the requirement for molecular oxygen, which elimin-

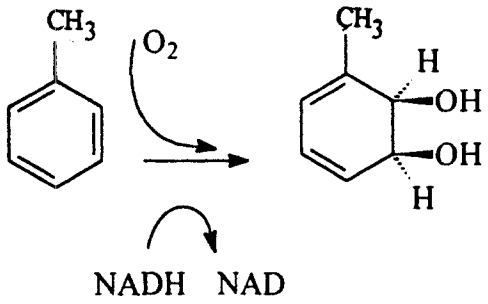

Figure 2 The oxidation of toluene to toluene-*cis*-glycol by *Pseudomonas putida* UV4.

ates the effective use of some reactor configurations. Earlier work [4, 18] demonstrated that when toluene was fed to an aqueous bacterial suspension, activity was lost rapidly if the aqueous toluene concentration rose above 2.4 mM, ie about 40% of the aqueous saturation value (5.5 mM, 0.5 g L^{-1}). The need to control the aqueous toluene concentration is critical, therefore, and has normally been achieved by careful control of the rate of feeding.

An alternative approach is to add a second, organic, phase [27] in which the toluene can be dissolved [7]. Using various criteria for selection, tetradecane was chosen as a solvent for toluene. Toluene has a much greater solubility in tetradecane than in water so it is possible to have high toluene concentrations in the reactor but prevent concentration in the aqueous phase from reaching a toxic level. The toluene *cis*-glycol production profile for a two-liquid phase biotransformation is compared with that for toluene-fed aqueous reaction in Figure 3. The cessation of product formation due to loss of activity by the bacteria was accompanied by a rise in the dissolved oxygen tension. Longer biotransformations with toluene feeding could be achieved using lower bacterial concentrations. Figure 3 illustrates that the constraints imposed by substrate toxicity and poor aqueous solubility can be overcome by a change in the reaction medium, which leads to a major increase in productivity. The *cis*-glycol product has a very high water

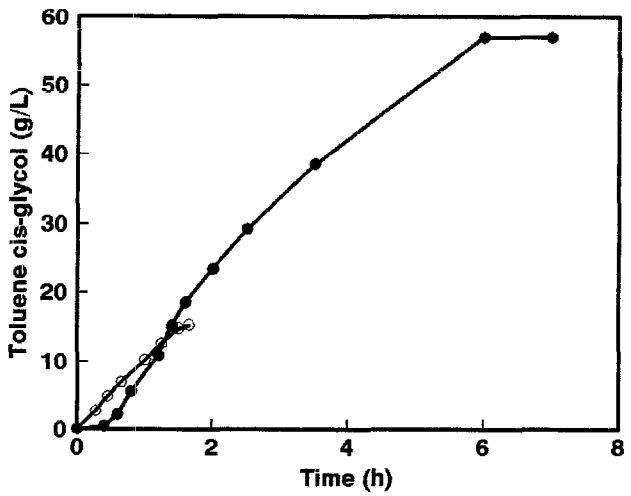

Figure 3 Comparison of the production profiles for toluene *cis*-glycol by *Pseudomonas putida* UV4 in a fed-batch stirred tank reactor with (●) and without (○) tetradecane present as a second liquid phase.

solubility and may be recovered from the aqueous phase. However, as indicated in Figure 1, there are inevitably interactions between the different parts of the process and in this case the introduction of a substantial organic phase, while benefitting the reaction, requires subsequent separation of the two liquid phases which may prove to be difficult if cell lysis has occurred.

Conversion of fluorobenzene to fluorocatechol by Pseudomonas putida ML2 [20,21]

Fluorobenzene is metabolised via fluorobenzene-*cis*-glycol to fluorocatechol by this bacterium (Figure 4). Steric hindrance afforded by the fluorine prevents further degradation by catechol 1,2-dioxygenase present in the cell [14]. In contrast to the toluene hydroxylation described above, where ethanol or another carbon source must be added to permit continued recycling of the oxidised cofactor (NAD), this conversion involves two enzymic steps, the second of which regenerates the cofactor. Nevertheless, ethanol was still added to the biotransformation to enhance metabolism of the bacteria. The reaction shares many characteristics with toluene hydroxylation, such as the requirement for molecular oxygen, poor aqueous solubility of the substrate (1.5 g L^{-1} for fluorobenzene) and the need to control the substrate concentration below a critical value. However, one critical difference is the toxicity of the product. Whereas toluene *cis*-glycol is non-toxic, fluorocatechol is toxic to the microorganism at concentrations above 0.2 g L^{-1}. This was a major constraint on satisfactory operation of the biotransformation, as less than 15% of the fluorobenzene that could be dissolved in an aqueous reaction medium could be converted before the rise in product concentration reached toxic levels [20]. This limitation was overcome by the use of *in situ* product removal (ISPR) [10].

Since fluorocatechol is water-soluble, extractive methods of ISPR were inappropriate and adsorptive techniques were considered. Whereas many adsorbents tested bound both fluorobenzene and fluorocatechol, fluorobenzene was preferentially bound to most of them. However, an activated carbon, Norit pK13, was chosen for product removal as with this material the binding of fluorocatechol was unaffected by the presence of fluorobenzene. Since fluorobenzene was fed to the biotransformation to ensure that it did not reach an inhibitory level, the amounts bound by the activated carbon were relatively low. This removed the need for a highly product-selective technique. The incorporation of a packed bed for *in situ* removal of fluorocatechol is shown diagrammatically in Figure 5. The bed (0.35 L volume) contained activated carbon particles (1–3 mm diameter). The recirculation rate (25.8 L h^{-1}) was designed to ensure that the fall in DOT before return of the bacterial suspension to the stirred tank reactor was not large enough to reduce the rate of biotransformation.

One remaining constraint was the loss of the volatile reactant as a result of air sparging of the reactor. This was overcome by omission of air sparging directly into the reactor and the introduction of a second circulation loop containing a membrane oxygenator (Figure 5), through which the bacterial suspension was pumped at 4.8 L min^{-1}. Using this system, very little fluorobenzene was lost from the reactor.

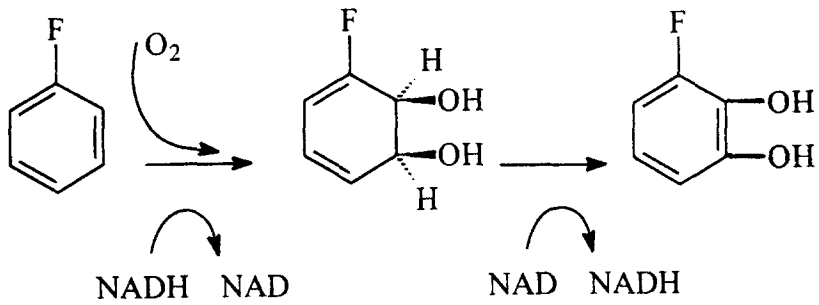

Figure 4 The conversion of fluorobenzene to fluorocatechol by *Pseudomonas putida* ML2.

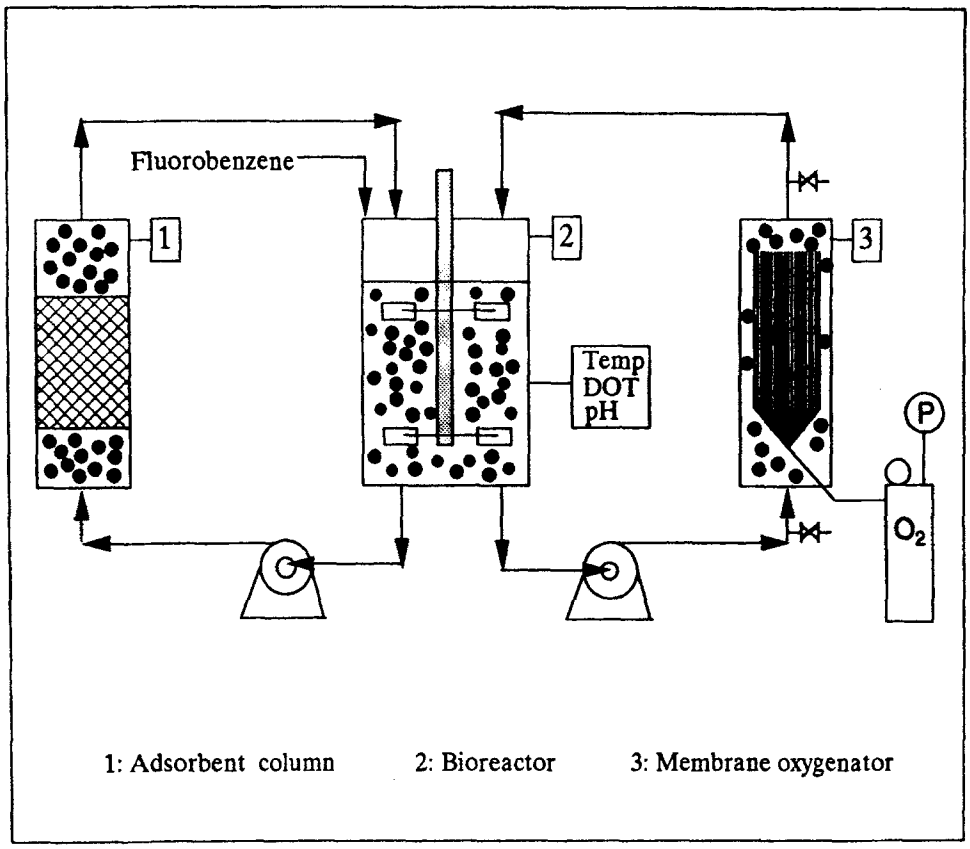

Figure 5 Diagram of reactor showing external circulation loops through a membrane oxygenator and packed bed for *in situ* product removal.

The results of a fed-batch biotransformation with both ISPR and membrane oxygenation are shown in Figure 6. A mixture of fluorobenzene and ethanol was fed for 14 h. During that time the biotransformation activity of the bacteria remained constant at 0.18 g fluorocatechol g dry wt^{-1} h^{-1}. At the end of the experiment the total amount of fluorocatechol produced was equivalent to 15 g L^{-1}, ie over seventy times greater than the minimum toxic level. The fluorocatechol was eluted from the activated charcoal by ethyl acetate.

Discussion

The development of a structured approach allows key decisions on the design and selection of biotransformation processes to be made more rapidly and effectively. This is important for the successful implementation of new or modified processes within industry. It also encourages biochemical engineers in industry to identify at an early stage the critical characteristics of the biotransformation of interest and the constraints imposed by these on process selection. Some characteristics are immutable (eg substrate and product properties) but others such as catalyst form and stability can be changed, by choice or by modification using recombinant DNA technology, to remove or alleviate a potential constraint. Here we have given examples of microbial oxidations where alternative modes of operation have overcome particular constraints—substrate toxicity, product toxicity and substrate volatility. For both biotransformations it has been possible to make beneficial step changes in productivity by either the introduction of a second, organic, liquid phase or *in situ* product removal.

The emphasis in this paper has been on the biotransformation itself but the structured approach based on evaluation

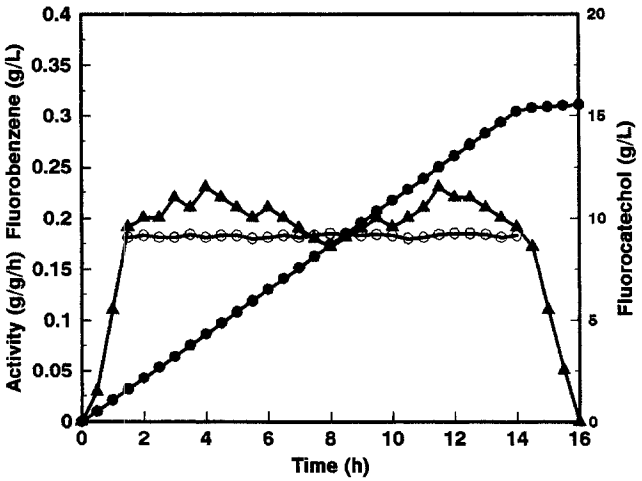

Figure 6 The production of fluorocatechol by *Pseudomonas putida* ML2 in a stirred tank reactor with recirculation of the bacterial suspension through a membrane oxygenator and a packed bed of activated charcoal. Fluorobenzene/ethanol (1:1, v/v) was fed to maintain the fluorobenzene concentration (▲) at about 0.2 g L^{-1}. Samples were assayed at intervals by biotransformation activity (○). The fluorocatechol adsorbed to the activated charcoal bed is expressed as an equivalent concentration (●) in the reaction mixture.

of the characteristics is also valuable in identifying constraints associated with biocatalyst production and product recovery. Whereas there is now a large number of publications on the use of microbial and enzymic catalysts, there are far fewer publications on the selection of the best conditions for their production. Recombinant DNA technology is already being used for enhancing catalyst production [12, 19] and activity, but further improvements in catalyst stability will result from the application of this technology.

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