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Use of free and immobilized *Pseudomonas putida* cells for the reduction of a thiophene derivative in organic media

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Abstract

An organic-aqueous two-liquid phase system was developed in order to perform the reduction of 3-thiophenecarboxaldeyde (3-TC) to 3-thiophenemethanol (3-TM), two intermediate log *P* compounds, using *Pseudomonas putida* S12 cells. Although, high bioconversion yields were observed in a bioconversion medium composed by Tris–HCl pH 8.5 buffer and *n*-octanol, adverse phase toxicity effects were observed following incubation periods in excess of 24 h. Furthermore, a stable emulsion was formed in two-liquid phase system in the presence of free cells. This prevented effective separation of the biocatalyst and of the two-liquid phases. Whole cells were effectively entrapped in Ca-alginate beads, thus enhancing biocatalyst recovery. No shifts in the pH versus activity profile resulted from the immobilization. Successive batches were carried out with the immobilized biocatalyst, but a decrease in the specific activity was observed.

Keywords: *Pseudomonas putida*; Thiophene derivative; Biocatalyst

1. Introduction

The use of whole cells as biocatalysts has become a valuable tool either for the production of a wide variety of compounds, from bulk to fine chemicals [1–3] or for bioremediation [4]. Many of these chemicals with potential economic interest are sparingly soluble in water and/or toxic to the microorganism. Thus, their addition to conventional aqueous media leads to ineffective bioconversion systems [3], due to their low productivity and to biocatalyst deactivation. Organic-aqueous two-liquid phase systems provide an attractive alternative to perform the bioconversion of such chemicals [5], once a biocompatible organic solvent, acting as substrate/product carrier, is selected. Hydrophobic solvents (log $P_{o/w} > 5$) are usually considered to comply with the biocompatibility criteria [6]. High substrate concentrations can therefore be used, leading to high volumetric productivities. Newly formed product can be extracted into the organic phase, thus allowing in situ product recovery and providing a first step for process integration. Toxic or inhibitory compounds can be kept at low concentrations in the aqueous phase [7–9], thus preventing their deleterious effect on the biocatalyst [10]. The non-polar nature of the biocompatible solvent may prove, on the other hand, relatively unsuitable for the extraction of intermediately polar products from the aqueous phase.

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The implementation of an effective organic-aqueous two-liquid bioconversion system for the production of such chemicals thus requires a relatively polar solvent to act as substrate reservoir and product sink [11]. Downstream processing can however be hampered, since stable emulsions are prone to be formed in organic-aqueous two-liquid biotransformation systems [12], namely due to cell lysis and concomitant release of membrane components [13]. Cell immobilization provides a tool to overcome such drawback and, furthermore it reduces toxicity effects [14–16], as compared to the use of free cells. The combination of organic-aqueous two-liquid–liquid phase systems and cell immobilization has been effectively used for the bioconversion of steroids [14,17], and in the production of dihyrodiols [18] and of flavor ketones [19].

In this work the feasibility of using an organic-aqueous two-liquid phase system to perform reduction of 3-thiophenecarboxaldeyde (3-TC) to 3-thiophenemethanol (3-TM), two intermediate $\log P_{o/w}$ compounds, using resting *Pseudomonas putida* S12 cells, was evaluated. These chemicals are useful intermediate in the production of cosmetics [20] and herbicides [21]. The profile of biocatalytic activity along the time course of the fermentation was evaluated. The effect of several operational parameters, namely pH of the aqueous phase, nature of the organic solvent used as carrier and the volumetric phase ratio, on the activity and stability of the biocatalyst were also evaluated. The effect of cell immobilization on biocatalytic activity was also studied.

2. Experimental

2.1. Microorganism and growth conditions

Pseudomonas putida S12 cells used in this study were obtained from Wageningen University, The Netherlands. Cells were cultured into 20 $g l^{-1}$ LB broth (Sigma, USA) slants, and either stored at 4 °C following 24 h growth at 30 °C or used to inoculate 250 ml erlenmeyer flasks containing 50 ml of LB broth medium (20 $g l^{-1}$). When an optical density of 1.2, measured at 640 nm was reached, cells were transferred to 2 l erlenmeyer flasks containing 500 ml of the same medium. In some trials, 3-thiophene carboxaldehyde (3-TC) was added to the growth medium in this latter production stage, to give a concentration of 1.2 or 20 mM. Incubation was carried out with 200 rpm orbital shaking at 30 °C. Cells were harvested by centrifugation along the time course of the fermentation or in the early lag phase (4 °C, 8000 rpm), washed with 20 mM potassium phosphate buffer pH 7 and stored at –20 °C until further use.

2.2. Partition trials

A given amount of substrate or product was dissolved in a chosen organic solvent and added to one volume of aqueous phase. The two phase system was equilibrated at 30 °C and samples from both phases were collected for HPLC analysis. Triplicates were run for each trial. P is defined as the partition of 3-TC or 3-TM between organic and aqueous phases.

2.3. Biocatalyst immobilization

A given amount of wet cell paste (2.0 g) was washed and ressuspended in 10 ml of the adequate Tris–HCl buffer, following centrifugation (4 °C, 8000 rpm) unless otherwise stated. This suspension was used to prepare several immobilized biocatalysts.

2.4. Calcium alginate beads

Immobilization procedure was adapted from [22]. The 400 μl of the cell suspension were mixed with 10 ml of a 4% (w/v) sodium alginate solution in water. The resulting solution was added dropwise to a 100 ml chilled solution of 0.18 mM calcium chloride. The resulting beads were hardened for 30 min at 4 °C, filtered through Whatman filter paper and used for bioconversion trials.

2.5. k-Carrageenan beads

The immobilization procedure was adapted from [23] and was the same as that with calcium alginate beads, but for the 30% (w/w) solution of *k*-carrageenan used, instead of sodium alginate solution, and for the hardening solution of 0.3 M KCl used.

2.6. Microencapsulation

The immobilization procedure was adapted from [24].The 400 μl of the cell suspension were added to 10 ml of a 1% (w/v) KCl solution, together with xanthan and PEG 400, in order to have a final concentration of 10% (w/v) and 1 gl^{-1}, respectively. This suspension was added dropwise to a 0.5% (w/v) sodium alginate solution, hardened for 30 min and washed with distilled water.

2.7. Adsorption to celite

An amount of 10 ml of a pH 8.5 Tris–HCl buffer (0.1 M) were added to 400 μl of the cell suspension, as well as 1 g Celite® R630 (Fluka, Switzerland) 20–230 mesh. The mixture was incubated with orbital shaking at 200 rpm and 30 °C for 2 h, and filtered through an adequate sieve. The immobilized biocatalyst was used for bioconversion.

2.8. Bead dissolution

Alginate beads were dissolved by incubation in 0.1 M sodium phosphate buffer pH 7 [23]. *k*-Carrageenan beads were dissolved by incubation in a 0.9% (w/v) NaCl solution [22]. Cells were recovered by centrifugation.

2.9. Bioconversion trials in two-liquid phase systems

A given amount of either free or immobilized biocatalyst was added to a given volume of 0.1 M Tris–HCl buffer pH 8.5, corresponding to 2.5 $g_{wet\ cell\ paste}\ l^{-1}$ or to 125 $g_{immobilized\ biocatalyst}\ l^{-1}$, respectively. One volume of a 12 mM solution of 3-TC in a water-immiscible organic solvent was added to the former and the whole incubated at 30 °C with 200 rpm orbital shaking or 450 rpm magnetic stirring. Orbital shaking experiments were carried out using 1 ml of emulsion contained in 4 ml screw capped vials, or 4 ml of emulsion in 25 ml screw capped vessels. Stirred tank experiments were performed using 10 ml of emulsion in a 20 ml volume screw capped vessel. Repeated batch production of 3-TM was started under the same conditions as for batch experiments. In these cases, the immobilized biocatalyst was ressuspended in fresh bioconversion medium, following a 24 h biotransformation run and 1 h incubation in growth medium. Free cells were harvested by centrifugation (12,000 rpm, 20 min). Duplicates were run for each trial. A 100 μl samples were collected periodically, centrifuged for phase separation and analyzed by HPLC. Triplicates were made for each trial. Samples from the emulsion were taken periodically, centrifuged for phase separation and analyzed by HPLC.

2.10. Bioconversion in aqueous phase system

A given amount of wet cell paste was washed and ressuspended in a 12 mM solution of 3-TC in 0.1 M Tris–HCl buffer pH 8.5, as to give a final concentration of 2.5 $g_{wet\ cell\ paste}\ l^{-1}$. Further procedures were similar to those described for bioconversion trials in two-liquid phase systems.

2.11. Catalytic stability tests

A given amount of free biocatalyst was ressuspended in pure or saturated 0.1 M Tris–HCl buffer pH 8.5, or in a *n*-octanol/0.1 M Tris–HCl buffer pH 8.5 (1:1 organic-aqueous volumetric phase ratio), and incubated at 30 °C with 200 rpm orbital shaking. Periodically, free cells were harvested by centrifugation (4000 rpm, 4 °C) and immediately used for bioconversion trials. Two independent runs were performed for each stability trial.

2.12. Analytical methods

HPLC (Merck-Hitachi, Germany) analyses were performed with a Lichrospher® 100 RP-18 column (250 mm × 4 mm; 5 μm particle diameter) (Merck, Germany) and isocratic elution, with water (35%) and ethanol (65%), substrate and product being detected at 245 nm, and matched to pure compounds (Aldrich, USA). The unit of catalytic activity (U) is equivalent to 1 mmol of 3-TM formed per minute. Errors in repeated experimental runs did not exceed 10%.

Protein was quantified by the Lowry method [25] following, if necessary, cell lysis, by heating at 100 °C for 10 min, in 1 M NaOH solution.

Biomass concentration was assessed, by evaluating the optical density of the cell suspension at 640 nm.

Dry cell weight was determined by drying at 80 °C, until constant weight.

3. Results and discussion

3.1. Biocatalyst production

In order to evaluate the activity profile along cell growth, the fermentation was monitored for assessment of specific activity and biomass concentration (Fig. 1). Biocatalyst activity increased along cell growth suggesting a primary metabolite-like behavior. The highest activity was thus observed at the end of the exponential phase. The reductive pathway of 3-TC showed no significant response towards the presence of this substrate in the fermentation media. The use of 3-TC as an inducer was thus discontinued (Fig. 1). Furthermore, a slight decrease in the specific growth rate, as well as in the final biomass yield, was observed when 3-TC concentration was increased to 20 mM. This suggested that substrate toxicity levels were reached. Further work was thus performed with *P. putida* cells grown in plain LB broth medium.

3.2. Screening of reaction solvents

The selection of the organic solvent used as substrate/product carrier in an organic-aqueous two-liquid phase system is based mainly on its biocompatibility towards the biocatalyst. However, the organic solvent used must also allow adequate partition of both substrate and product, in order to provide a pool for those chemicals. Homologous series of *n*-alkanols and *n*-alkanes were assayed for their effective use in the implementation of a biotransformation medium, based on those criteria (Table 1). The concentration of substrate used in these trials (12 mM), as well as that of product formed, is well below solubility limits (data not shown), thus preventing phase saturation. As shown in Table 1, although each *n*-alkane tested is less toxic to the biocatalyst than the corresponding *n*-alkanol, according to their respective log $P_{o/w}$ values, the latter class of solvents proved more suitable for use as separate organic phase, since higher product yields were obtained. Furthermore, due to the unfavorable partition, the product formed in the presence of *n*-alkanes was not extracted into the organic phase. Thus, the lower overall product yields observed in these latter trials could also be related to some degree of toxic action of the product formed towards the biocatalyst. The reduction promoted by the biocatalyst requires a reducing co-factor [26]. The *n*-alcohols used could also provide a supplementary source for co-factor recycling, through its oxidation to the corresponding aldehyde, along with the continuous recycling system ensured by the use of the whole cell system [26]. The oxidation of ethanol to acetaldehyde was used for NAD/NADH co-factor recycling system when a cell-free crude extract of baker's yeast

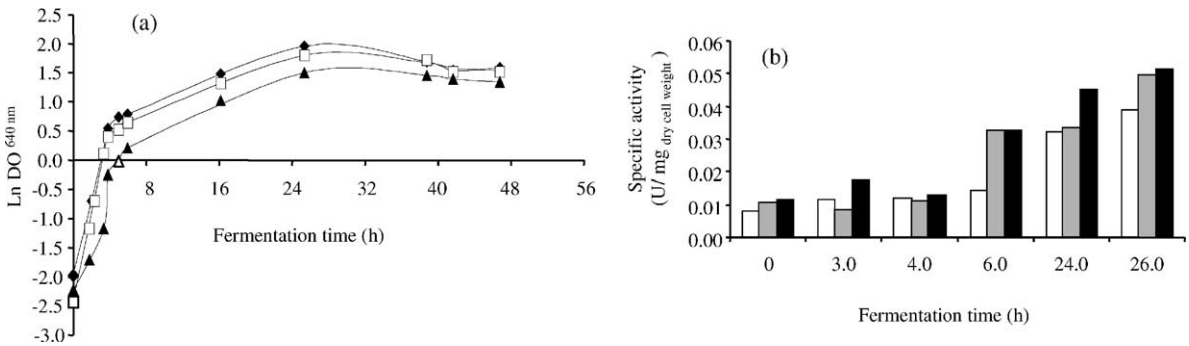

Fig. 1. Typical graph (a) of the growth of *Pseudomonas putida* S12 in 20 mM potassium phosphate pH 7 buffered LB broth media, either plain (◆), or containing 1.2 mM 3-TC (□) or 20 mM 3-TC (▲). Specific biocatalytic activity for the reduction of 3-TC to 3-TM along fermentation time is depicted (b), when cells were grown in plain LB broth medium (□), or in the presence of 1.2 mM 3-TC (▩) or 20 mM 3-TC (■). Bioconversion trials were performed in an organic-aqueous two-liquid phase system, at pH 8.5 and with 400 rpm orbital shaking. A 12 mM solution of 3-TC in *n*-octanol was used as organic phase.

Table 1
Parameters evaluated for the screening of reaction solvents

Solvent	Log $P_{o/w}$ solvent[a]	Product yield in organic phase (%)	Total product yield (%)	Log P 3-TC	Log P 3-TM
n-Hexanol	1.82	11.1	14.8	1.13	0.97
n-Octanol	2.81	34.6	46.2	1.03	0.82
n-Decanol	3.79	21.8	33.1	0.91	0.72
n-Dodecanol	4.77	12.4	23.1	0.86	0.67
Hexane	3.29	0	13.4	0.38	−1.03
Octane	4.27	0	16.0	0.37	−1.05
Decane	5.25	0	14.1	0.37	−1.11
Dodecane	6.23	0	13.6	0.38	−1.11

Hydrophobic nature of the organic solvents, used as a second phase in bioconversion and partition trials, expressed as $\log P_{o/w}$ in a standard octanol–water system was considered, as well as total product yield and product yield in organic phase. Bioconversion was carried out for 30 min, at 30 °C, with 400 rpm orbital shaking. Partition data of the substrate (3-TC) and of the product (3-TM), in the trialed organic solvents, expressed as $\log P$, in an organic-aqueous two-liquid phase system, at 30 °C, is also shown.

[a] Theoretical data obtained from the Syracuse Research Corporation database (http://esc.syrres.com/interkow/kowdemo.htm).

was used for benzaldehyde reduction to benzyl alcohol [27]. Also according to the data, and with the exception of *n*-hexanol, an increase in product yield is observed when $\log P$ 3-TC increases. Thus, a more favorable partition to the aqueous phase leads to a decrease in product yield, suggesting a deleterious effect on the biocatalytic pathway. On the other hand, the decrease in product yield observed when *n*-hexanol was used as organic phase can be ascribed to solvent toxicity. Among the organic solvents evaluated, *n*-octanol provided the best compromise between low toxic action on the biocatalyst and adequate partition properties. The effectiveness of *n*-octanol as a discrete organic phase for the integrated bioproduction of slightly hydrophilic chemicals was also evidenced in the production of 3-methylcatechol ($\log P_{o/w} = 1.58$) [11]. Nevertheless, the presence of the organic phase was not totally harmless towards the biocatalyst, and its effect following long incubation periods can be easily assessed (Fig. 2). According to the data presented, it is clear that molecular toxicity effects of the solvent on the biocatalyst can be excluded. On the other hand, the presence of a discrete organic phase led to protein leakage, most probably due to cell membrane damage. The deleterious effect of water-immiscible organic solvents on the membrane structure of *P. putida* cells was also observed by Kim and Rhee [13], during the growth of *P. putida* 3SK in

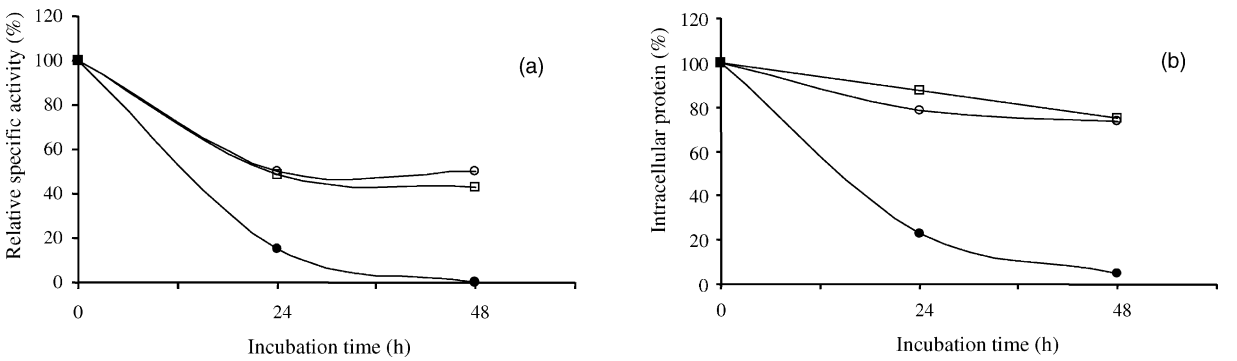

Fig. 2. Catalytic stability (a) based on the rate of product formation along incubation time of *P. putida* S12 cells when incubated in pure Tris–HCl buffer (○), in saturated buffer (□) or in a *n*-octanol: Tris–HCl buffer two-liquid phase system (●). Protein concentration (b) within the pellet remained approximately constant, except for two-phase system, where an 80% decay was observed.

the presence of isooctane, and by Schmid et al. [28], during the growth of *P. oleovorans* in the presence of *n*-octane. The similarity of the protein composition of the aqueous supernatant and the cell pellets, reported by the latter authors, further suggested cell lysis as the cause for the high level of proteins in the aqueous supernatant [28]. In the present work, residual catalytic activity for the reduction of 3-TC to 3-TM was detected in the supernatant, therefore also suggesting cell lysis. The presence of a discrete organic phase was nevertheless necessary in order to enhance the accumulation of 3-TM, suggesting deactivation of a competing metabolic pathway evidenced when an aqueous reaction system was used, according to data related to substrate metabolization (Fig. 3). During bioconversion trials in aqueous phase, HPLC analysis showed the presence of an unidentified peak (data not shown). The corresponding area consistently increased along with the time course of the bioconversion time, whereas the substrate concentration decreased and some 3-TM was formed. Shifting of the pH of the reaction media was shown to be a key factor in the manipulation of degradative pathways for the production of aromatic aldehydes [29]. In the present work, pH shifting clearly influenced biocatalyst activity (Fig. 3), however it was not determinant for specificity.

The organic to aqueous phase ratio had a marked influence on the specific reduction activity of *P. putida* cells (Fig. 4). Since the total cell load and organic phase concentration were kept constant, the observed trend in the catalytic activity could be related to a variation in the interfacial area, which depends on the volumetric phase ratio, as observed by Áscon-Cabrera and Lebeault [30]. Therefore, a decrease in the interfacial area would make the substrate less accessible to the biocatalyst, hence reducing the observed activity.

3.3. Biocatalyst immobilization

Biocatalyst immobilization is often coupled to organic-aqueous two-liquid phase systems in order to ease recovery steps, as well as to minimize deleterious effects of the organic phase in the biocatalyst [10]. Several immobilization methods were compared in order to assess their possible use in the system studied (Fig. 5). After a 24 h bioconversion trial, it was observed that the entrapment methods allowed a significant retention of the biocatalyst within the beads, based on intracellular protein. Celite, on the other hand, did not prove quite effective in the retention of biocatalyst, suggesting that the interactions between support and cells are too weak.

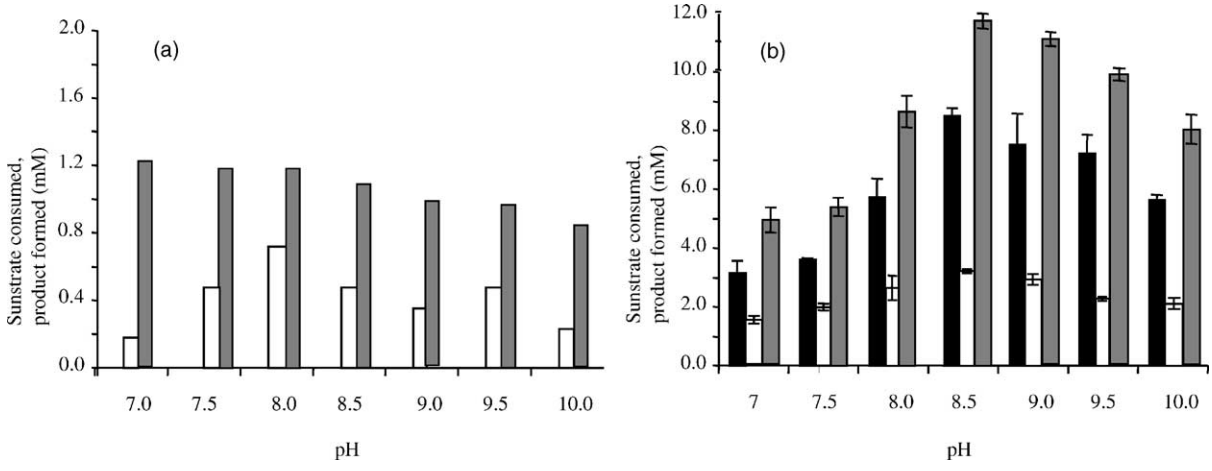

Fig. 3. Microbial reduction of 3-TC in aqueous (a) and organic-aqueous two-liquid phase systems (b), as a function of the pH of the bioconversion medium. Total substrate consumed (▩) and product formed in the aqueous (□) and in the organic (■) phases are shown. In aqueous phase trials a 12 mM solution of 3-TC in 0.1 M Tris–HCl buffer was used. In emulsion systems, Tris–HCl 0.1 M was used as buffer and a 12 mM solution of 3-TC in *n*-octanol was used as organic phase. Bioconversion trials were carried out at 30 °C, with 200 rpm orbital shaking using 4 ml emulsions kept in 25 ml screw-capped flasks. Samples were taken after a 24 h run.

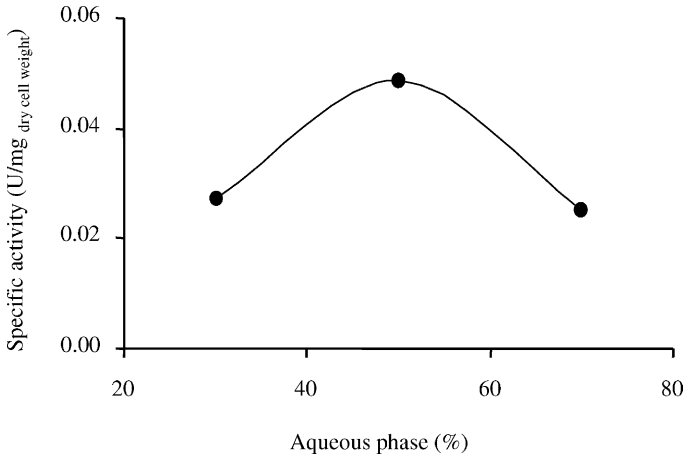

Fig. 4. Influence of aqueous to organic phase ratio on the specific activity of reduction of 3-TC to 3-TM. Tris–HCl 0.1 M was used as buffer and a 12 mM solution of 3-TC in *n*-octanol was used as organic phase. Bioconversion trials were carried out at 30 °C, with 450 rpm magnetic stirring.

Irrespective of the immobilization method used, no evidence of an unwanted side reaction was observed, since product formation was in accordance with substrate metabolization (data not shown), suggesting that *n*-octanol partitions into the beads. Otherwise, and since the immobilization methods used are mild, and thus not prone to induce cell damage, it could be expected that unwanted catalytic activity could be present, particularly when entrapment methods were used. Thus, the lower apparent activity observed in *k*-carrageenan beads and alginate microcapsules, as compared to Ca-alginate beads, could be related to unfavorable mass transfer.

The specific reduction activity versus pH profile for Ca-alginate immobilized cells shows an optimum at pH 8.5 (Fig. 6), which is similar to that observed when

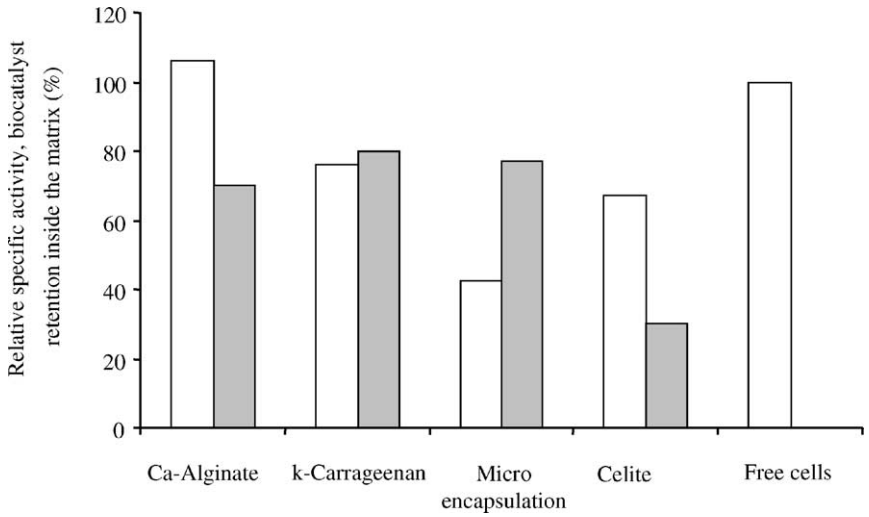

Fig. 5. Effect of the immobilization method on biocatalyst retention (▩) and reduction activity (□) compared to the results with free cells, after a 24 bioconversion run in an organic-aqueous (pH 8.5) two-liquid phase system, at 30 °C with 200 rpm orbital shaking. *P. putida* cells were immobilized in Celite, Ca-alginate, *k*-carrageenan or microencapsulated in Ca-alginate beads. The 0.5 g immobilized biocatalyst were used in this trial.

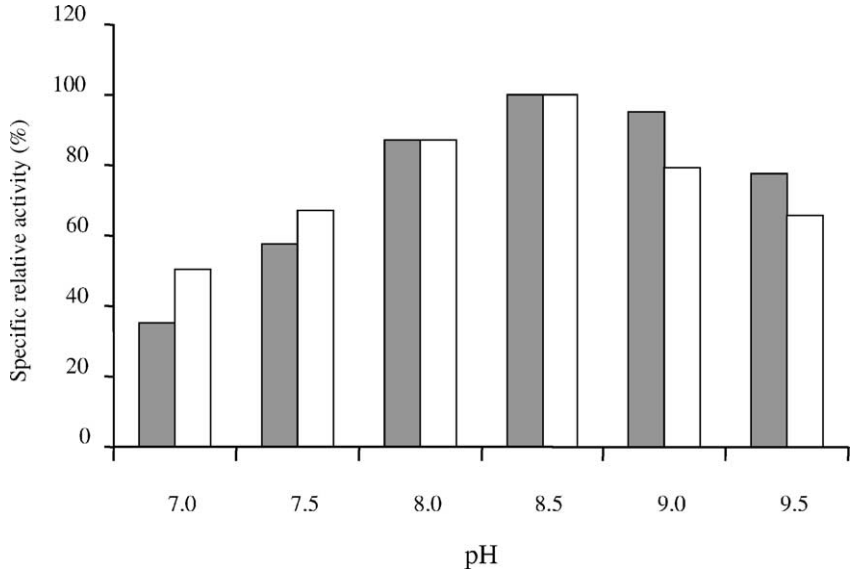

Fig. 6. Effect of the pH of the biotransformation medium in the relative specific activity of free (■) and Ca-alginate entrapped (□) *Pseudomonas putida* S12 cells for the production of 3-TM. Biotransformation was carried out in an organic-aqueous two-liquid phase system, at 30 °C and with 200 rpm orbital shaking.

free cells were used. Besides, no relevant changes are observed when comparing the activity profiles in both systems, further suggesting minor changes in the microenvironment of the immobilized form of the biocatalyst, as compared to free cells.

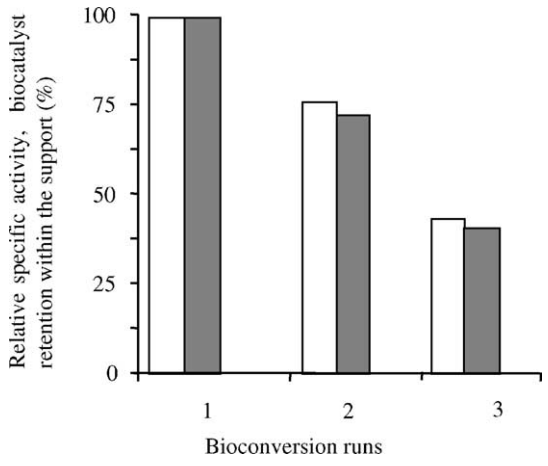

Fig. 7. Repeated batch bioconversion of 3-TC to 3-TM using Ca-alginate entrapped *P. putida* cells in an organic-aqueous two-liquid phase system. Relative specific activity (□) and biocatalyst retention within the matrix are shown (■).

Cell immobilization eased the recovery of the biocatalyst from the bioconversion medium and allowed its use in repeated bioconversion trials (Fig. 7). Although there is still protein leakage, no catalytic activity was determined in the aqueous supernatant, suggesting either a protective effect of the immobilization matrix or the retention of the enzyme complex responsible for the reductive catalytic activity within the matrix pores.

4. Conclusions

Pseudomonas putida S12 cells present biocatalytic activity for the reduction of 3-thiophenecarboxaldehyde to 3-thiophene methanol, higher activity levels being observed towards the end of the exponential phase. The presence of a discrete organic phase prevents unwanted side-reactions, *n*-octanol providing the best compromise between biocompatibility and partition of substrate and product, among the solvents tested. However, a phase toxicity effect became evident, following long term exposure to the solvent. Biocatalyst entrapment in Ca-alginate beads provided the best compromise between biocatalyst retention

and apparent activity, as compared to entrapment in *k*-carrageenan or microencapsulation in alginate, or adsorption to Celite. The optimum pH for biocatalytic activity was 8.5, irrespective of the use of free or immobilized cells and unwanted side reactions are absent, which suggests minor changes in the microenvironment of the biocatalyst. Cell immobilization allowed the use of the biocatalyst in repeated biotransformation trials. However, the reduction in specific catalytic activity, together with some protein leakage, observed in the successive batches prevented the effective use of this approach.

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