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ORIGINAL PAPER

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# Optimization of isonovalal production from $\alpha$-pinene oxide using permeabilized cells of *Pseudomonas rhodesiae* CIP 107491

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**Abstract** Optimization studies on the synthesis of isonovalal from $\alpha$-pinene oxide by *Pseudomonas rhodesiae* CIP 107491 operated in a biphasic medium are presented. Three key parameters are identified. The first is the need for a permeabilization of cells by freezing them and then treating the thawed material with an organic solvent such as chloroform, toluene or diethyl ether. This operation allows both enzyme release into the aqueous phase outside the cells and an improvement in the transport properties of both substrate and product across the cell membrane, strongly increasing reaction rates. The second is that the enzyme $\alpha$-pinene oxide lyase, which exhibits an irreversible inactivation by isonovalal (or a by-product), presents a constant turn-over, i.e., the total product synthesis is proportional to the biomass loading and is close to 108 mmol (16.4 g) isonovalal $l^{-1}$ $g^{-1}$ biomass. The third phenomenon is that the biphasic system used is not phase-transfer-limited, a feature attributed to the spontaneous formation of an oil-in-water emulsion. It is thus possible to carry out a very efficient process, allowing the recovery of 2.63 mol isonovalal $l^{-1}$ (400 g $l^{-1}$) from 25 g biomass $l^{-1}$ in 2.5 h, corresponding to an average reaction rate as high as 0.70 mmol $min^{-1}$ $g^{-1}$ cells (160 g $l^{-1}$ $h^{-1}$).

## Introduction

The microbial cell membrane acts as an osmotic barrier and, most often, precursors and products involved in biotransformation processes move across this structure by passive diffusional transport. When intact microbial cells are used in biocatalysis, therefore, the cell membrane may severely hamper a bioconversion from working optimally because substrate influx and product efflux by diffusion can be slow with regard to the maximal reaction rate (van der Werf et al. 1992, 1995, 1996).

For these reasons, cells used in commercial bioconversions are often permeabilized, in order to improve the rate of exchange between intra- and extra-cellular media. Several methods have been described for this purpose (Felix 1982). Many enzymes from Gram-negative bacteria can be extracted by the use of water-immiscible solvents, such as toluene or chloroform, which can cause disruption of the cell membranes, resulting in enzyme leakage (Jackson and DeMoss 1965; Helenius and Simons 1975). Microbial cells can also be effectively broken by physical means. Sonication is convenient and effective on the laboratory scale, while high-pressure extrusion equipment, such as the French press, is currently the method of choice for industry (Cabral 2001).

In a recent publication (Fontanille et al. 2002), a new bacterial strain, *Pseudomonas rhodesiae* PF1 (CIP 107491), was described and reported to catalyze the cleavage of both rings of $\alpha$-pinene oxide to form the Z form of 2-methyl-5-isopropyl-hexa-2,4-dien-1-al (isonovalal, Fig. 1). Studies of $\alpha$-pinene metabolism from *P. fluorescens* NCIMB 11671 (Best et al. 1987; Burfield et al. 1989; Colocousi et al. 1996) or *Nocardia* sp. P18.3 (Griffiths et al. 1987a, b) reported that the enzyme catalyzing this reaction, $\alpha$-pinene oxide lyase, lacked prosthetic groups and had no cofactor requirement. All authors also noticed a limited stability of the enzyme, which could hamper the economic feasibility of this process.

In this paper, we report some results obtained during the course of optimization of the above process, which enabled the design of a very fast system, allowing the recovery of about 2.63 mol isonovalal $l^{-1}$ (400 g $l^{-1}$) after only 2.5 h reaction.

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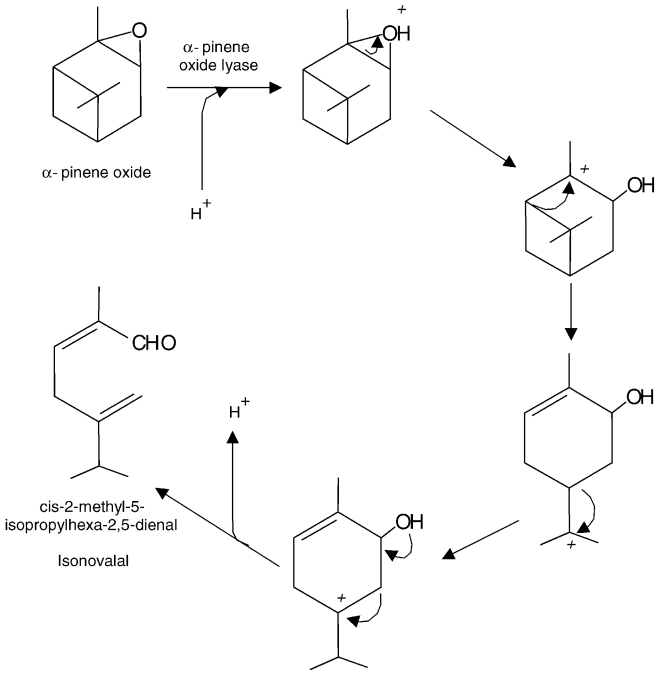


**Fig. 1** Mechanism proposed for $\alpha$-pinene oxide decyclization by *Nocardia* sp. strain P18.3 lyase (from Griffiths et al. 1987a, b). Intermediates were not detected in this work

## Materials and methods

### Microorganism strain preservation

*P. rhodesiae* strain PF1 (CIP 107491) has been deposited in the Collection de l' Institut Pasteur (Fontanille et al. 2002). It is a gram-negative rod able to grow with $\alpha$-pinene as sole carbon source. *P. rhodesiae*, which belongs to the fluorescent group of *Pseudomonas* was isolated from natural mineral water (Coroler et al. 1996). This strain was maintained on nutrient agar (Difco) poured in Petri dishes held at 30 °C. After 24 h growth, the dishes were stored at 4 °C for 3–4 weeks before replication. A stock material was also prepared using the Protect bacterial preserver system (Technical Service Consultants, Heywood, UK), which is made of porous ceramic beads immersed in a cryopreservative fluid stored at –75 °C (Feltham et al. 1978; Pàlagyi et al. 1997).

### Preculture in Erlenmeyer flasks

Erlenmeyer flasks of 500 ml volume, containing 250 ml *Pseudomonas* basal medium (Cohen-Bazire et al. 1957) and 5 g sodium lactate $l^{-1}$ as carbon source (Pequignot et al. 1998), were inoculated with colonies picked out on an agar plate (see above). After 24 h growth at 30 °C in a rotary shaker operated at 200 rpm, the optical density at 600 nm ($OD_{600}$) of the media was close to 3 (biomass concentration 1.2 g $l^{-1}$). These broths were then used to inoculate the Erlenmeyer flasks or the bioreactor.

### Biocatalyst production

Biocatalyst production was carried out in an aerated, stirred bioreactor (Biostat ED; B. Braun, Melsungen, Germany) of 5 l working volume. The medium was made of 4 l *Pseudomonas* basal medium with sodium lactate being replaced by glucose (5 g $l^{-1}$) and 200 ml of an organic solution made of 8.5 ml $\alpha$-pinene in hexadecane (10.6 mmol $l^{-1}$). A 320-ml sample of the preculture (see above) were used as inoculum. The stirring rate was maintained at 500 rpm and the temperature and aeration rate were 30 °C and 30 l $h^{-1}$ (0.11 vvm), respectively. The pH was allowed to decrease from its initial value (7.00) to 6.00 and was then maintained using 1 mol NaOH $l^{-1}$. The process was stopped after 9–12 h cultivation.

The resulting broth was then centrifuged for 10 min at 2,600 *g* and ambient temperature. The pelleted cells obtained were resuspended in an appropriate volume of phosphate buffer (20 mmol $l^{-1}$, pH 7.5) to yield an $OD_{600}$ of 16, corresponding to a biomass concentration close to 7.2 g $l^{-1}$ (the relationship between biomass and optical density was established as $X=0.447\times OD_{600}$). This concentrated suspension was stored frozen at –20 °C.

### Biomass permeabilization

The above cell suspension in phosphate buffer was thawed and poured into a 500-ml Erlenmeyer flask. The appropriate amount of permeabilizing agent (5% for the organic solvents, toluene, chloroform or diethylether; 1% for the detergent, Triton X-100) was added to the aqueous suspension. In each case, the cell permeabilization process was allowed to proceed for 1 h at 30 °C, the flask being placed in a rotary shaker operated at 200 rpm.

Physical treatment by sonication was applied to a thawed concentrated suspension using a sonicator (Ikasonic U50H) at a maximal ultrasonic energy of 125 W $cm^{-1}$. Three cycles of 30 s, at 1 min intervals, were carried out for all samples.

### Biotransformation performance

The reaction medium was a two-phase system comprising an aqueous phase made of the thawed concentrated cell suspension (permeabilized or not) in phosphate buffer and an organic layer that consisted of an organic solution of $\alpha$-pinene oxide in hexadecane. Initial substrate (hexadecane) concentrations ranged from 0.66 mol $l^{-1}$ to 3.95 mol $l^{-1}$, the phase volume ratio was 1:1, and reaction temperature was 30 °C. The vessels used were either 250-ml Erlenmeyer flasks or a stirred bioreactor of 1.2 l working volume (Applikon Dependable Instruments, Schiedam, The Netherlands; Doig et al. 1998). Flasks were filled with 50 ml medium (25 ml of each phase) and placed in a rotary shaker (200 rpm). Sequential additions of precursor were performed in order to avoid any substrate limitation. Typically, they were done hourly, using a 0.26 mol $l^{-1}$ solution of $\alpha$-pinene oxide in hexadecane, the volume added each time being close to 1 ml. The same procedure was applied to the bioreactor, which contained 500 ml medium, with about 10 ml of the above substrate solution added at 30-min intervals. The stirring rate was 800 rpm, the system was not aerated, and the pH was not controlled.

When sequential biomass feedings were performed, the bioreactor was used and each addition, performed at 2-h intervals, corresponded to the same amount of thawed and permeabilized biocatalyst as that used at the beginning of the reaction. However, suspensions used in this case were ten-fold concentrated, which allowed only 25 ml of aqueous solution to be introduced to the system.

### Fed-batch bioconversion in a monophasic medium

This bioconversion was performed in the same bioreactor and under the same conditions of stirring and temperature as those previously described. The medium consisted of 400 ml thawed and permeabilized cell suspension in phosphate buffer. $\alpha$-Pinene oxide was added continuously to the reactor by means of a peristaltic pump, at a rate of 0.13 mol $l^{-1}$ $h^{-1}$. This value allowed an instantaneous precursor consumption, which avoided any organic phase formation.

**Table 1** Experimental parameters achieved during the course of $\alpha$-pinene oxide biotransformation after different methods of biomass permeabilization. Experiments were carried out in Erlenmeyer flasks (see Materials and methods) with 7 g biomass $l^{-1}$. The initial rate was measured after 30 min of bioconversion. *Supernatant* Experiment was carried out with the supernatant obtained after centrifugation of frozen biomass further pemeabilized with diethyl ether (see Materials and methods). – Not determined

| Experiment number | Biocatalyst form and treatment | Initial rate of isonovalal production ($\mu mol^{-1}$ $min^{-1}$ $g^{-1}$ cells) | Maximal isonovalal synthesis (mmol $l^{-1}$) | Yield of isonovalal production (mol $mol^{-1}$) | Concentration of protein in supernatant after treatment (g $l^{-1}$) |
|---|---|---|---|---|---|
| 1 | Fresh cells | 27 | 79 | 0.30 | 0.41 |
| 2 | Fresh cells + toluene | 370 | 474 | 0.63 | – |
| 3 | Frozen cells | 500 | 480 | 0.60 | 1.48 |
| 4 | Frozen cells + toluene | 1,140 | 750 | 0.81 | 1.69 |
| 5 | Frozen cells + chloroform | 1,270 | 783 | 0.77 | 1.69 |
| 6 | Frozen cells + diethyl ether | 1,280 | 776 | 0.81 | 1.71 |
| 7 | Frozen cells + Triton X-100 | 1,750 | 757 | 0.83 | 2.82 |
| 8 | Frozen cells + sonication | 1,190 | 711 | 0.82 | 1.68 |
| 9 | Supernatant | 1,065 | 561 | 0.75 | 1.71 |

Biotransformations analysis

The organic phase was directly analyzed by gas chromatography (GC) by injecting 1 µl of an aliquot sample into the apparatus. Volatile (hydrophobic) compounds in the aqueous layer were extracted with a hexane–diethyl ether mixture (volume ratio 1:1), the resulting organic solution being subjected to gas–liquid analysis. The GC (HP 5890; Agilent Technologies, Palo Alto, Calif., USA) was fitted with an apolar SPB-5 (Supelco, Bellefonte, Pa., USA) capillary column (30 m × 0.32 mm i.d., film thickness 0.25 µm) and a flame ionization detector. The carrier gas was nitrogen and the oven temperature was kept at 80 °C for 5 min and then raised to 220 °C by 20 °C $min^{-1}$. The injector and detector temperatures were 250 °C for both; and the split ratio for injection was 1:5. Concentrations were calculated by assuming that all compounds had the same response factor as $\alpha$-pinene oxide. The internal standards were heptadecane for samples from organic layers and methyl nonanoate for aqueous phase extracts. Heptadecane was already present as an impurity in hexadecane, which made useless any addition of this compound in samples to be analyzed. The use of a molecule already present ensured that its concentration was always the same (for a given lot number). It was then possible to draw calibration curves directly from the ratio of the area of the peak of interest to that of heptadecane, which made unnecessary the knowledge of the exact concentration of this last compound.

Products obtained were identified by GC-mass spectral analysis (GC-MS). The samples were separated using an apolar HP-5MS (Agilent Technologies) capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The carrier gas was helium and other operating parameters were as above. The GC was a Agilent 6890-plus, which was coupled to a 5973 N MS-engine. MS conditions were an electron-impact ionization energy of 70 eV, an accelerating voltage of 1.1 kV, an emission current 35 µA, a quadrupole, ion source, and interface (line transfer) temperatures of 150 °C, 230 °C and 280 °C, respectively. Library searches were performed using the NIST/EPA/NIH MS library (version 1.7a, 2000).

## Results

### Influence of biomass permeabilization treatment on biotransformation

The positive effect of the freezing–thawing procedure on the bioconversion efficiency was demonstrated in a recent study (Fontanille et al. 2002). This treatment led to a large increase in both reaction rate and yield of isonovalal. Moreover, a significant enzymatic activity was recovered in the supernatant resulting from the centrifugation of frozen cells, which was not the case with fresh ones. It was concluded that diffusional phenomena across the cell membrane produced a limiting step in this system. The influence of different organic solvents suitable for cell permeabilization (Felix 1982), such as diethylether, chloroform or toluene, was therefore studied with the aim of improving the system. Detergents, such as Triton X-100, and physical treatment, like biomass sonication, were also used.

Results obtained with fresh cells showed that organic solvents had the same effect on the biocatalyst as that observed with frozen biomass (see experiments 1–3 in Table 1). These compounds led to a further improvement, both in terms of maximal isonovalal synthesis and initial reaction rate, when they were used with previously frozen cells (experiments 3–6 in Table 1). This effect appeared to be the same for all three solvents used and also for sonication (experiment 8 in Table 1), while Triton X-100 provided the best yield.

More detailed analysis of these data revealed that organic solvents and sonication gave a minor increase in protein release from cells (Table 1). It was also noticed that a significant enzyme activity remained attached to the cells, since the maximal isonovalal synthesis was lowered when only supernatant was used (see experiments 6, 9, Table 1). It could thus be assumed that their main effect

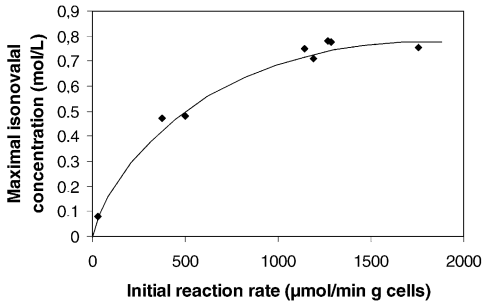


**Fig. 2** Maximal isonovalal concentration plotted against initial reaction rate for different ways of biomass permeabilization, from data in Table 1. Experimental conditions as in Table 1. *L* Liters of hexadexane

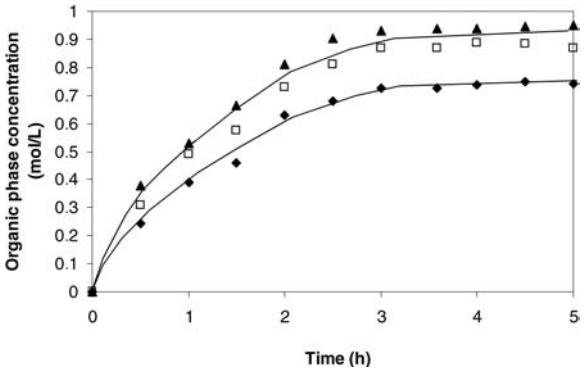


**Fig. 3** Typical time-course of $\alpha$-pinene oxide consumption (*black triangles*), total products (*white squares*) and isonovalal production (*black diamonds*) during bioconversion of $\alpha$-pinene oxide with cells (7 g $l^{-1}$) previously permeabilized with diethylether. The experiment was performed in an Erlenmeyer flask at 30 °C and 200 rpm, with a phase volume ratio of 1:1

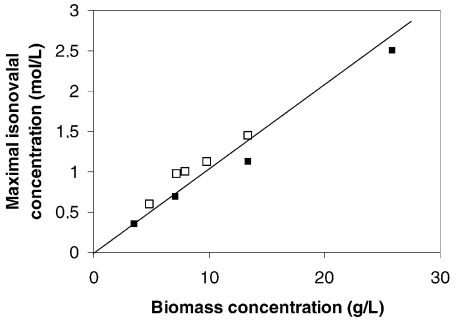


**Fig. 4** Maximal isonovalal concentration obtained plotted against biomass concentration. Experiments were carried out in Erlenmeyer flasks (*white squares*) or in a bioreactor (*black squares*) in a biphasic system (hexadecane/phosphate buffer) with a diethyl ether-permeabilized biomass

on previously frozen biocatalyst was an improvement in the transport properties of $\alpha$-pinene oxide and isonovalal across the cell membrane. Triton X-100 gave rise to a more pronounced membrane alteration, allowing additional protein (enzyme) release outside the cells.

It was also noticed that, as previously reported (Fontanille et al. 2002), the biotransformation process always stopped, even in the presence of precursor, due to enzyme inactivation. This feature allowed the determination of the maximal isonovalal concentration achieved for a given biomass concentration (7 g $l^{-1}$). This parameter gave a curve showing a plateau for permeabilized frozen cells, when it was plotted against the initial reaction rate (Fig. 2). These conditions thus corresponded to a maximal enzyme stability and enabled the recovery of ca. 0.76 mol isonovalal $l^{-1}$, using 7 g biomass $l^{-1}$.

Biotransformation media took the form of an oil-in-water emulsion. The two phases could be separated by centrifugation (see Materials and methods) in order to allow analytical procedures to be carried out. It was also found that this separation could be easily performed by settling when the aqueous layer pH was increased to a value of 11 by NaOH addition. However, this approach did not work when Triton X-100 was present in the medium. This feature led us to avoid the use of this detergent and only frozen cells permeabilized with an organic solvent were used in the following work. Figure 3, where the substrate is given as the amount consumed, shows a typical time-course of experiments carried out in this way.

## Biomass concentration

The straight line in Fig. 4 demonstrates that the maximal isonovalal concentration was proportional to the biocatalyst content. The same feature was observed with the initial reaction rate (data not shown). These parameters took similar values for both experimental designs used, i.e., Erlenmeyer flasks and stirred bioreactor operated at 800 rpm (see Materials and methods). Experiments carried out in the bioreactor at stirring speeds ranging over 600–1,200 rpm gave the same results (data not shown).

It could thus be considered that, surprisingly, Erlenmeyer flasks were able to give results that could be easily scaled-up. This feature was, obviously, due to the fact that the biphasic system used in this study was not limited by mass transfer between phases, but by the amount of active enzyme present.

These features also showed that the enzyme inactivation took place in a similar way whatever the biomass concentration and that it was possible to obtain ca. 108 mmol isonovalal $l^{-1}$ $g^{-1}$ biomass (from the slope of the line in Fig. 4). As a result, 25 g biomass $l^{-1}$ gave a final isonovalal concentration as high as 2.63 mol $l^{-1}$ solvent in about 2.5 h reaction (Fig 5).

## Influence of the nature of the organic phase

The biocatalyst, although based on the use of whole cells, was in fact mainly made of solubilized enzyme. It could thus be expected that it could be operated with organic

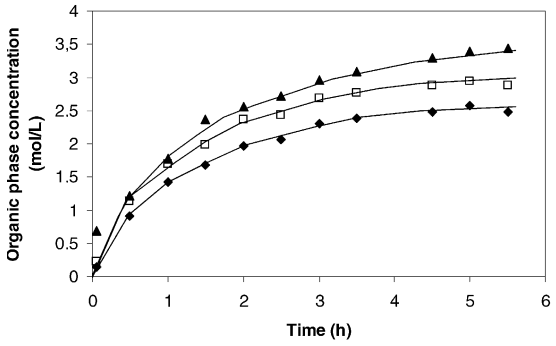


**Fig. 5** Typical time-course of $\alpha$-pinene oxide consumption (*black triangles*), total products (*white squares*) and isonovalal production (*black diamonds*) during the biotransformation of $\alpha$-pinene oxide in a biphasic system (hexadecane/phosphate buffer) using a high biomass loading. Cells (26 g $l^{-1}$) were permeabilized with diethyl ether and the experiment was carried out in a bioreactor at 30 °C and 800 rpm, with an initial $\alpha$-pinene oxide concentration of 2.53 mol $l^{-1}$ and further addition of substrate (1.26 mol $l^{-1}$) after 1 h

solvents exhibiting a Log P (logarithm of the partition coefficient in a standard water–octanol system; Weber and de Bont 1996) less than that of hexadecane. It was thus decided to try solvents with lower boiling points, such as hexane and isooctane, in order to facilitate product purification.

Results obtained confirmed the high stability of the enzyme system towards organic solvents, since the final isonovalal content was always close to 0.66 mol $l^{-1}$, with a biomass loading of 6.5 g $l^{-1}$.

## Characterization of enzyme inactivation

The above experiments always showed an enzyme inactivation, leading to the isonovalal synthesis stopping. Several attempts were made to characterize this phenomenon.

Stability of the enzyme in the biphasic system was checked. No activity loss occurred when permeabilized cells were allowed to stand for 24 h in the biotransformation medium before the introduction of $\alpha$-pinene oxide. This behavior was in agreement with the fact that an experiment carried out in an homogeneous (aqueous) system (see Materials and methods) gave a time-course similar to that observed in the standard biphasic medium. Here again, the biotransformation process stopped after about 2.5 h (data not shown).

Also, the use of an inactivated aqueous layer, resulting from a terminated reaction process, with a fresh organic phase did not allow any further biotransformation. This feature occurred even after intensive washings of the aqueous medium with fresh hexadecane. It was thus demonstrated that this inactivation was an irreversible process.

Further, tests were carried out with active cells in contact with organic layers resulting from previously performed reactions, thus containing isonovalal and minor

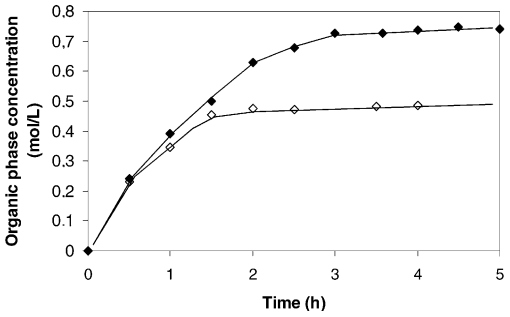


**Fig. 6** Different time-course of isonovalal production during the bioconversion of $\alpha$-pinene oxide in biphasic systems with cells (7 g $l^{-1}$) permeabilized with diethyl ether. The organic phase was made of fresh hexadecane (*black diamonds*), or hexadecane containing bioconversion products and isonovalal (0.75 mol $l^{-1}$; *white diamonds*). Experiments were carried out in Erlenmeyer flasks

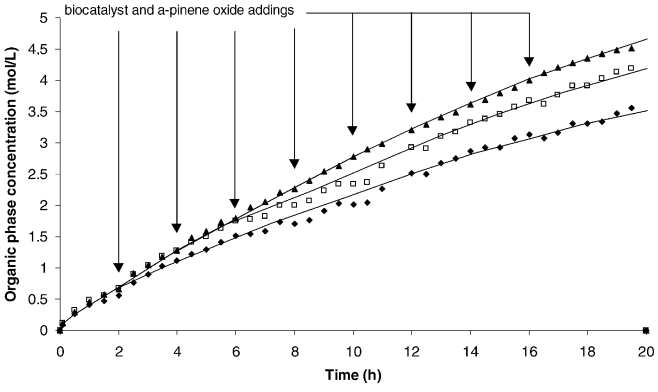


**Fig. 7** Time-course of $\alpha$-pinene oxide consumption (*black triangles*), total products (*white squares*) and isonovalal production (*black diamonds*) during bioconversion of $\alpha$-pinene (*a-pinene*) oxide carried out in the bioreactor with sequential addition (*addings*) of cells permeabilized with diethyl ether. The initial biocatalyst content was 7 g $l^{-1}$ and further biomass feeds (7 g $l^{-1}$) were performed every 2 h with 25 ml concentrated suspensions containing 70 g biological material $l^{-1}$

amounts of by-products, but being precursor-free. When the experiment was started immediately after building the medium, the process stopped prematurely (Fig. 6). When the precursor was added 24 h later, no biocatalytic activity ($\alpha$-pinene oxide consumption or isonovalal production) could be detected. It should be noted that no change in the organic phase took place during the 24-h delay. As above, subsequent washings of the aqueous layer were unable to re-activate the biocatalytic system. It was thus concluded that inactivation was a time-dependent process, connected not to the reaction itself but to the presence of isonovalal (or a by-product).

Finally, an experiment, carried out with a double fed-batch technique, including precursor (in the usual manner) and biocatalyst loadings at 2-h intervals (see Materials and methods), allowed us to obtain 3.29 mol isonovalal $l^{-1}$ in 20 h (Fig. 7). However, a continuous decrease in the reaction rate was observed, despite adding active enzyme. This result showed that the inactivation

process was also dependent on the inhibitor concentration.

## Discussion

The biotransformation process studied involved non-purified $\alpha$-pinene oxide lyase, produced by a recently isolated bacterium, *P. rhodesiae* (Fontanille et al. 2002). A key parameter, from a process point of view, was to achieve good contact between the precursor and the enzyme. Since no cofactor was needed, it was possible to use resting cells without respiratory activity. Furthermore, membrane permeabilization allowed a dramatic improvement in enzyme release outside the cells, allowing very fast biotransformation processes to take place. A very simple protocol can be proposed, that involves the use of polar solvents which are also compatible with the biotransformation itself. This demonstrates a high enzyme stability towards compounds with a Log P as low as 0.89 for diethyl ether (Hansch et al. 1995).

This material had obviously the same main biocatalytic features as those already reported for this kind of well documented enzyme (Trudgill 1994). The main common feature was that it exhibited a "suicidal catalysis" (Trudgill 1994), generally considered to result from the reaction itself, which involves the donation of a proton from a site in the catalytic center (see Fig. 1). Results obtained in this work showed this phenomenon was actually irreversible and could be observed even in the absence of a biocatalytic process, when the enzyme was in contact with isonovalal and side-products. This phenomenon clearly demonstrated that at least one of these compounds was able to interact with the enzyme. One hypothesis could be that a binding to the protein could occur, yielding changes in the active-site conformation, which would become unable to perform the above-mentioned proton donation, initiating the biocatalytic process. This phenomenon was not instantaneous and was dependent on the inhibitor concentration. However, it was possible to recover ca. 3.29 mol isonovalal $l^{-1}$, with sequential biomass (enzyme) feedings, after 20 h reaction.

Another important point was the fact that, in optimized conditions, a constant turn-over could be defined for the enzyme, corresponding to the production of 108 mmol isonovalal $l^{-1}$ $g^{-1}$ biomass. This parameter allowed the calculation of the biocatalyst loading necessary for a wanted level of production. Furthermore, it appeared that the reaction rate increased proportionally to biocatalyst loading, indicating this system was enzyme-limited, at least in the experimental conditions assayed in this work. In other words, no phase-transfer limitation could be shown. This was quite surprising, because such limitation could occur even with reaction rates considerably lower (Grivel et al. 1999; Grivel and Larroche 2001). This behavior probably resulted from the fact that the biphasic medium appeared to be an oil-in-water emulsion, thus giving a large exchange area (Gargouri 2001). These features gave an extremely efficient process, since it was possible to recover about 2.63 mol $l^{-1}$ (400 g $l^{-1}$) in 2.5 h reaction, corresponding to an impressive average synthesis rate close to 0.70 mmol $min^{-1}$ $g^{-1}$ cells (160 g $l^{-1}$ $h^{-1}$ with a cell loading of 25 g $l^{-1}$).

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