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Solvent selection for the biotransformation of terpenes by *Pseudomonas putida*

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Abstract

A two-phase reaction system for the biotransformation of terpenes was implemented in order to protect the microorganism from the toxic effects of substrates, intermediates and products and to prevent the oxidation of the compounds by oxygen and/or water. Preliminary studies were carried out with a *Pseudomonas putida* wild strain. Growth of the wild strain was not affected by the presence of 0.5% (v/v) α-pinene. However, the presence of α-pinene oxide in concentrations above 0.2% (v/v) had a progressive inhibitory effect on growth (complete inhibition occurred at 0.5%). Since substrate and/or product toxicity can often be overcome by the use of an organic solvent reservoir, we searched for a biocompatible solvent. Solvents with a log P close to 4.5 were the most effective in minimising α-pinene oxide toxicity, especially 1-dodecanol, as judged from growth and oxygen uptake experiments. Although the growth rate was 25% lower in the presence of 2% (v/total volume) of organic phase, growth and oxygen consumption rates were not further affected by the introduction of 0.5% (v/total volume) of α-pinene oxide in the solvent phase. The substrate α-pinene itself (log P 4.9) was tested as a solvent reservoir for α-pinene oxide and found effective.

Keywords: Terpenes; Two-phase reaction system; Biocompatibility; log P value; Biotransformation

1. Introduction

The microbial transformation of readily available and low-priced monoterpenes, such as α-pinene, has a considerable potential interest for application in the flavour and fragrance industries. Nevertheless, the difficulties encountered in the biotransformation of these compounds are very complex. Many microorganisms selected through screening and adaptation often transform or degrade the added terpenoid into a variety of metabolites which might be useless or difficult to separate (van der Werf et al. [1]). Toxicity of monoterpenes to the whole cells (Sikkema et al. [2]), instability and low solubility of substrates and/or products in water are additional problems posed by these biotransformations. It is the purpose of the E.C. project 'Terpene biotransformations' to construct single product producing strains either by cloning genes of interest from terpene converting microorganisms into strain *Pseudomonas putida* NCIMB 8248, that is unable to transform terpenes, or by knocking out undesired pathways in terpene transforming strains. Main attention is currently focused on the transformation of

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α-pinene through pinene oxide into borneol. The genes responsible for the α-pinene monooxygenase have already been identified and isolated by D. Leak and co-workers [3]. This research group is currently attempting to express these genes in *P. putida*. The present article addresses the development of a microbial reaction system able to transform α-pinene to α-pinene oxide. The approach chosen was the introduction of an organic solvent as reservoir for the substrate and the product. Removing terpenoids from contact with the aqueous phase is expected to improve the stability of these compounds (pinene oxide is very unstable in water), while preventing their toxicity towards the biocatalyst. One very important criterion in the selection of an organic solvent is its biocompatibility, a measure of which is given by the log *P* value, defined as the logarithm of the solvent partition coefficient in a standard octanol/water two-phase system (Laane et al. [4]). According to these authors, solvents with a log *P* above 4 are generally non-toxic. *P. putida* NCIMB 8248 wild strain was used as representative of the behaviour of the future recombinant strain with regard to growth in the presence of terpenes and/or organic solvents. The objective of this study is to show that the toxicity problems associated with this biotransformation model reaction can be minimised through the implementation of appropriately designed reaction systems.

2. Materials and methods

2.1. Microorganism

P. putida wild strain NCIMB 8248 was obtained from the Department of Biochemistry of Imperial College, London, U.K.

2.2. Maintenance and growth

The microorganism was maintained on agar slants containing *Pseudomonas* isolation agar (Difco). The strain was grown on 150 ml of *Pseudomonas* basal medium (Cohen-Bazire et al. [5]) using L-glutamic acid monosodium salt (GA) as carbon source (0.2% (w/v), except when indicated). Biomass was cultivated in 1L shaken flasks in an orbital incubator at 30°C and 300 rpm. Growth experiments were started by inoculating with 4.5 ml of an overnight culture grown in the same medium. For growth in the presence of monoterpenes, flasks were closed with a rubber bung and various initial concentrations of the compounds were added to the culture medium. For growth in the presence of an organic reservoir, 2% (v/v) of the solvent was added. When testing the organic solvent as a reservoir for α-pinene oxide, 0.5% (v/v) of the monoterpene was added to the solvent phase. Growth was followed by measuring the OD (600 nm). When solvents and/or terpenes were present, the sample was first centrifuged and resuspended in phosphate buffer before determining the OD (600 nm). These values were later converted in dry weight concentrations (g/l) using a calibration curve.

2.3. Chemicals

Solvents tested for use as an organic phase were cyclohexane (> 99.5%), *n*-hexane (> 95%), *n*-dodecane (> 99%), 1-dodecanol (> 98%), purchased from Merck, and *iso*-octane (99.5%) from Riedel de-Haën. The terpenes used were (±)α-pinene (98%) and α-pinene oxide (97%), from Aldrich Chemicals.

2.4. Oxygen consumption rate

The strain was grown on GA (0.2% (w/v)) at 300 rpm. Cells were harvested at the end of the exponential phase and washed twice with a 50 mM phosphate buffer, pH 7. The biomass was resuspended in the same buffer and stored at 4°C. Initial oxygen consumption rate was measured in a 4 cm^3 reaction chamber fitted with an oxygen electrode (Hansatech Instru-

ments) at 30°C. The medium consisted of 2 ml air-saturated 50 mM phosphate buffer, pH 7, containing 0.5% (w/v) GA. The electrode was calibrated at the beginning of each experiment by measuring the electrode signal of the air-saturated media before adding the cells. Experiments were started by injecting 15–25 μl of the cell suspension, resulting in a final OD (600 nm) of about 0.16–0.20. Each experiment was carried out at least in triplicate and was alternated with a blank measurement (i.e., measurement of oxygen consumption rate by cells in the absence of solvent and/or terpene). Initial oxygen consumption rate was expressed as gO_2 consumed/(g DW.h).

3. Results and discussion

3.1. Growth in the presence of terpenes

The toxicity of α-pinene and α-pinene oxide towards growing cells was tested by growing cells in the presence of concentrations up to 0.5% (v/v) of the terpenes without a solvent reservoir (Table 1).

Increasing concentrations of pinene oxide had a progressive inhibitory effect on growth, complete inhibition occurring at 0.5% (v/v). Growth in the presence of 0.5% (v/v) α-pinene proceeded close to the blank.

Table 1
Final biomass attained in batch cultures of *P. putida* NCIMB 8248 grown on 0.1% (w/v) GA in the presence of different concentrations of α-pinene and α-pinene oxide

Terpene	Concentration (% v/v)	Dry weight (g/l)
–	–	0.36
α-Pinene	0.05	0.36
	0.50	0.33
α-Pinene oxide	0.05	0.34
	0.11	0.35
	0.20	0.35
	0.30	0.13
	0.40	0.12
	0.50	0.06

Table 2
Effect of the presence of 2% (v/total volume) of organic phase on the final dry weight of cultures of *P. putida* NCIMB 8248 grown on 0.1% (w/v) GA

Organic solvent	log *P*	Dry weight (g/l)
–	–	0.36
Cyclohexane	3.2	0.20
n-Hexane	3.5	0.26
iso-Octane	4.5	0.28
n-Dodecane	6.6	0.32

Cell cultivation was also attempted in the absence of GA. It was observed that none of the terpenes supported growth.

3.2. Growth in the presence of organic solvents

We made a preliminary selection of solvents which were expected to be biocompatible on the basis of their log *P* value. Since gram-negative bacteria, and especially *Pseudomonas*, have been described as more tolerant to organic solvents than other microorganisms (Vermüe et al. [6]), we also tested the biocompatibility of some organic solvents with lower log *P* values which could be advantageous in terms of price. In fact, solvents with a high log *P* hampered growth to a lesser extent as compared to solvents with a low log *P* (Table 2).

3.3. Growth in the presence of an organic phase containing α-pinene oxide

From the solvents tested we chose those which had less inhibitory effects upon growth to act a reservoir for α-pinene oxide. These were *n*-dodecane and *iso*-octane (Table 2). At this stage, we decided to assay also 1-dodecanol as a potential organic phase because its polarity is an indication of its better ability to dissolve the epoxide, as compared to the apolar medium-chain hydrocarbons. On the basis of the very low toxicity shown by α-pinene (Table 1), we also attempted to find out whether it could be used as the organic phase, thus avoiding the introduction of a third component in the culture medium.

Table 3
Growth of *P. putida* NCIMB 8248 on 0.2% (w/v) GA with 2% (v/total volume) of organic solvent as a reservoir for 0.5% (v/total volume) α-pinene oxide

Organic solvent	log *P*	Presence of α-pinene oxide	Final dry weight (g/l)	Duration of lag phase (h)	Growth rate (h^{-1})
–	–	no	0.64	–	0.91
iso-Octane	4.5	no	0.62	0.6	0.76
		yes	0.55	0.7	0.89
α-Pinene	4.9	no	0.55	1.2	0.53
		yes	0.65	1.8	0.70
1-Dodecanol	5.0	no	0.78	1.2	0.69
		yes	0.85	0.6	0.80
n-Dodecane	6.6	no	0.87	2.8	0.64
		yes	0.61	0.8	0.76

The introduction of an organic solvent considerably prolonged the lag phase. In the case of 1-dodecanol and *n*-dodecane, the duration of this lag phase decreased when pinene oxide was present. A positive effect on growth rate was noticed when α-pinene oxide was added to any of the organic solvents. We did not find an explanation for these phenomena. The inhibitory effects upon growth caused by α-pinene oxide were overcome by the presence of any of the organic phases (Table 3). This was probably a result of the absence of a dispersed phase of pinene oxide, preventing direct contact of the cell with the oxide.

3.4. Oxygen consumption of resting cells

It is still to be determined whether the process format of the biotransformation of α-pinene will be single-stage (fermentation and biotransformation carried out in the same bioreactor) or two-stage (fermentation followed by cell separation and subsequent use of cells in the production reactor). In the latter case reuse of cells possibly requires maintenance of viability, since cofactor regeneration might be a prerequisite for continued biotransformation activity. Thus, it is important to measure the oxygen consumption rate of resting cells as an indication of their viability. Two of the solvents used, namely *n*-dodecane and *iso*-octane, interfered with the oxygen measurements. However, the values obtained with these solvents allowed us to conclude that no significant decrease of oxygen consumption rate occurred upon introduction of pinene oxide. Results in Table 4 clearly indicate that 1-dodecanol was the solvent which less affected the respiration rate of resting cells.

Table 4
Relative initial oxygen consumption rate of resting cells of *P. putida* NCIMB 8248 (grown on 0.2% (w/v) GA) in the presence of 2% (v/total volume) of organic phase containing or not α-pinene oxide[a]

Organic solvent	log *P*	α-pinene oxide (% v/v)	Relative respiration activity (%)
–	–	0.05	25
–		0.20	0–10
iso-Octane	4.5	–	31–41
α-Pinene	4.9	–	53
		0.20	47
1-Dodecanol	5.0	–	97
		0.2	98
n-Dodecane	6.6	–	45–72
		0.2	38

[a]100% activity ranged between 0.35 and 0.70 g O_2 consumed/(g DW.h), depending on the physiological state of the cell suspension.

4. Conclusions

The substrate, α-pinene, of our model reaction did not affect the growth rate of *P. putida* NCIMB 8248 on GA in concentrations up to 0.5% (v/v). However, the product, α-pinene oxide, seriously inhibited bacterial growth and cellular respiration of *P. putida*. Selective removal of this product would overcome these problems, but is difficult to achieve due to its similarity to the substrate. However, the toxicity problems associated with our biotransformation model reaction could be minimised through the implementation of an appropriate aqueous/organic reaction system. According to the kinetic and respiration data (cf. Tables 3 and 4), 1-dodecanol was found to be the solvent of choice to overcome α-pinene oxide toxicity towards *P. putida* cells. The substrate α-pinene itself was tested as a solvent reservoir for α-pinene oxide and found effective. The organic/aqueous phase ratio will be optimised in future work.

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