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Integrated bioproduction and extraction of 3-methylcatechol

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Abstract

Pseudomonas putida MC2 is a solvent-tolerant strain that accumulates 3-methylcatechol. In aqueous media, 10 mM of 3-methylcatechol was produced and production was limited by 3-methylcatechol toxicity to the biocatalyst. Production levels increased by introduction of a second, organic phase that provides the substrate toluene and extracts the product from the culture medium. Octanol was shown to be an appropriate second phase with respect to tolerance of the strain for this solvent and with respect to partitioning of both substrate and product. Per unit of overall reactor volume (octanol and water), best results were obtained with 50% (v/v) of octanol: an overall 3-methylcatechol concentration of 25 mM was reached with 96% of the product present in the octanol phase. These product concentrations are much higher than in aqueous media without organic solvent, indicating that biocatalysis in an organic/aqueous two-phase system is an improved set-up for high production levels of 3-methylcatechol.

Keywords: *Pseudomonas putida* MC2; 3-Methylcatechol production; Optimization; Organic/aqueous two-phase system; Phase ratio

1. Introduction

In biocatalysis, many processes are limited by inhibition of substrates and/or products (Robinson et al., 1992; Collins et al., 1995; Marshall and Woodley, 1995; Bont, 1998). Cells start to produce the desired compound, but after a while they are inactivated because the product reaches a toxic level. In case the substrate is toxic, cells require a low initial substrate concentration.

Substituted catechols are valuable precursors in pharmaceutical production processes (Ennis and Ghazal, 1992; Scharrenburg and Frankena, 1996; Hartog and Wouters, 1988), but they are difficult to synthesize chemically (Scharrenburg and Frankena, 1996; Held et al., 1999). We studied the

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model conversion of toluene to 3-methylcatechol by *Pseudomonas putida* MC2 in aqueous media (Hüsken et al., 2001a,b). For this bioconversion, growing cells are needed. Growth stopped early during this process, due to the rising product concentration, and production was therefore limited.

There are several ways to overcome substrate and product inhibition. In situ product removal, for example with an adsorbent column, can improve reaction conditions for the biocatalyst. Lilly and Woodley (1996) validated this approach for the conversion of fluorobenzene to 3-fluorocatechol by *P. putida* ML2 by adsorbing substrate and product onto activated carbon. Robinson et al. (1992) used a similar product removal system in 3-methylcatechol production from toluene by *P. putida* 2313; a packed bed with granular activated charcoal removed the product. Held et al. (1999) described the production of 3-phenylcatechol from 2-phenylphenol by *Escherichia coli* JM101 (pHBP461). In their integrated process, substrate and product were adsorbed on Amberlite™ XAD-4.

A second means to overcome substrate and product inhibition and to enhance production is the use of an organic phase as substrate reservoir and product sink (Brink and Tramper, 1985; Harbron et al., 1986; Tramper et al., 1992; Collins et al., 1995; Bont, 1998; Collins and Daugulis, 1999a,b; Wery et al., 2000). Exchange kinetics and optimization of organic/aqueous systems have been studied by Cesário et al. (1996, 1997). However, many bacteria are intolerant to otherwise appropriate organic solvents (Cruden et al., 1992; Harrop et al., 1992; Vermuë et al., 1993; Isken et al., 1999).

Toxicity of organic solvents for micro-organisms depends on the hydrophobicity of the solvent (Weber and Bont, 1996). Log $P_{o/w}$ values denote the tendency of a compound to partition over a two-phase system of octanol and water (Rekker and de Kort, 1979; Vermuë et al., 1993); the higher the log $P_{o/w}$, the better the compound dissolves in octanol and the more hydrophobic it is. Most bacterial cells do not survive in the presence of a second organic phase with a log $P_{o/w}$ value in-between 1 and 4 (Bont, 1998).

The log $P_{o/w}$ of 1-octanol is 2.92 (Osborne et al., 1990), indicating its toxicity (Bont, 1998). *P. putida* DS10 (derived from *P. putida* S12), however, was tolerant to this solvent and was shown to be capable of producing 3-methylcatechol in a 10 ml scale octanol/aqueous two-phase system (Wery et al., 2000).

In 3-methylcatechol production, partitioning of substrate and product are in favor of octanol. Substrate toluene (log $P_{o/w} = 2.60$; Osborne et al., 1990) dissolves 400 times better in octanol than in water, while product 3-methylcatechol (log $P_{o/w} = 1.58$; Meylan and Howard, 1995) dissolves 38 times better in octanol.

In this paper, we show that *P. putida* MC2, a recombinant derived from *P. putida* F1, a strain with a relatively high solvent-tolerance, thrives in the presence of 1-octanol. Optimal bioproduction of 3-methylcatechol as depending on the octanol fraction is defined by: (1) a high overall 3-methylcatechol concentration; (2) the fraction of product that is present in the octanol phase for downstream processing; and (3) the total process time to produce the acquired amount of product. *P. putida* MC2 is shown to be capable of producing high amounts of 3-methylcatechol in an octanol/aqueous two-phase system, both at 10 ml and at 0.80 l scale. Overall product concentrations were found to be high as compared to aqueous production systems and depended on the ratio between the organic and the aqueous phase.

2. Materials and methods

2.1. Pseudomonas putida MC2

P. putida MC2 was previously constructed and characterized (Hüsken et al., 2001a). The strain originally contained a natural toluene degradation pathway under control of a toluene promoter. This native pathway was mutated such that enzymatic degradation of the product was not possible anymore. Into this strain, additional sets of *todC1C2BAD* genes for 3-methylcatechol production and a kanamycin resistance gene were introduced. The additional *todC1C2BAD* genes were under control of a salicylate promoter.

2.2. Media

Solid media were used for overnight precultures from −80°C glycerol stocks; they contained 2% of agar and 50 μg of kanamycin per ml of LB medium (Sambrook et al., 1989).

LB broth was used as complete medium. Toluene (0.5 mM) was added to liquid precultures for induction of the native genes for toluene degradation and 3-methylcatechol accumulation. Cells were grown at 30°C without an antibiotic. For 3-methylcatechol production studies, the strain was pregrown to a cell density of 0.60 g l^{-1} on the basis of cell dry weight (logarithmic phase), measured as the optical density at 660 nm (OD_{660}).

2.3. 3-Methylcatechol production

At $t = 0$, sodium salicylate (1.0 mM) was added to the incubation media for induction of the genes involved in 3-methylcatechol formation by *P. putida* MC2. The reaction substrate toluene was added to such an extent that an initial aqueous concentration of 1.0 mM was established. In water–air systems, the main amount of toluene was present in the gas phase (Amoore and Hautala, 1983). Final product concentrations could therefore be considerably higher than initial aqueous toluene concentrations. The partition coefficient of toluene over air and water, 1:3.8 (Amoore and Hautala, 1983), and the air:water volume ratio were used to calculate the required total amount of toluene.

For two-liquid-phase incubations, liquid toluene was supplemented together with solvent 1-octanol to the aqueous, salicylate-containing medium at $t = 0$. Cells were not pre-adapted to the solvent. Desired toluene concentrations for two-liquid-phase systems were calculated from known $\log P_{o/w}$ values and phase ratios. According to the $\log P_{o/w}$ value of toluene, 2.60 (Osborne et al., 1990), the octanol phase should contain 400 mM of toluene to obtain a concentration of 1.0 mM of toluene in the aqueous phase at equilibrium. The equilibrium toluene concentration in the gas phase was 0.26 mM (Amoore and Hautala, 1983).

2.4. Small-scale experiments

Small-scale experiments were carried out in airtight Boston bottles (Phase Separations), containing 10 ml of liquid and 240 ml of air. These experiments were batches without aeration or pH control. The bottles were incubated in a horizontally shaking waterbath at 30°C.

Organic and aqueous medium together had a volume of 10 ml in small-scale experiments, while their ratio varied. The large air volume was chosen to minimize oxygen limitation.

2.5. Fermentations

The bioreactor used was an Applikon 1.8 l flat-bottom fermentor (Applikon Dependable Instruments, Schiedam, The Netherlands) and contained 0.80 l of total liquid. 0, 20, 40 or 50% (v/v) of this volume consisted of the organic phase 1-octanol. Octanol contained 400 mM of toluene, resulting in an aqueous concentration of 1.0 mM of toluene if equilibrium between octanol and aqueous medium is assumed.

The medium contained 2‰ (v/v, based on aqueous medium) of antifoam agent polypropylene glycol (average MW 2000) and was aerated at 200–400 ml min^{-1}. Clean air was mixed with toluene–saturated air, resulting in air containing toluene at 18% saturation. Since the maximum solubility of toluene in water is 5.6 mM (Osborne et al., 1990), at 18%, 1.0 mM of toluene is maintained in the aqueous phase by feeding this air to the reactor, assuming toluene degradation was slower than toluene transport.

The pH of the aqueous medium was maintained at 7.0 with 1.0 M of HCl. The oxygen tension was monitored to manually control agitation rates. At inoculation, the agitation rate was 200 rpm. After 1 min it was increased to 400 rpm and when the oxygen tension dropped, a higher rate was applied. The maximum applied agitation rate was 800 rpm for an aqueous system and 500 rpm for a two-phase system.

Samples of approximately 3 ml were taken through a sample port. A 50 ml syringe and 0.2 μm filter were used to push air through the sample port, to get rid of stagnant zones inside the sam-

ple pipe. After that, a sample was extracted from the reactor.

2.6. Analytical methods

Cell densities in aqueous systems were measured in a spectrophotometer (Perkin-Elmer lambda 2 UV/VIS spectrophotometer), at 660 nm (OD_{660}) using 1.0 cm plastic cuvettes (Greiner). The product did not interfere with this measurement as long as the broth color did not turn brown. In two-phase systems, it was not possible to use this method for measuring cell densities; here CO_2 concentrations were measured in headspace (small scale) or off gas (bioreactor) to monitor cell activities. CO_2 concentrations were measured in 100 μl headspace samples in a model 1530A gas chromatograph (Hewlett Packard HP6890 Series GC System) with a Poraplot Q column (Chrompack). Toluene concentrations were measured in 100 μl headspace samples in a similar gas chromatograph with a 10% SE-30 chromosorb WMP column (Chrompack).

Samples for 3-methylcatechol measurement were centrifuged in an Eppendorf centrifuge (Heraeus Instruments, Biofuge Fresco) at 13000 rpm and 4°C for 3 min. Maximally 200 μl of the aqueous phase supernatant was used in a colorimetric determination at 510 nm of 3-methylcatechol (Arnow, 1937) (extinction coefficient in LB medium 1.65 mM^{-1} cm^{-1}).

Using this same analysis, the partition coefficient for 3-methylcatechol over octanol and LB medium was measured. In a known quantity of LB medium, 3-methylcatechol was dissolved and the Arnow method was used to measure the product concentration (Arnow, 1937). After that, an equal quantity of octanol was added. The volume was vortexed for 15 min at 30°C and the aqueous product concentration was again measured. From these two concentrations, the partition coefficient was determined.

As a control, occasionally product concentrations in octanol were determined. An HPLC was used with a C18 column of 250 × 4.6 mm (L × ID; Chrompack Chromsep, Zorbax 7), applying a flow of 1.2 ml min^{-1} of eluents, containing 0.1% trifluoracetic acid in a 1:1 methanol–water matrix. 3-Methylcatechol was detected in a UV spectrophotometer at 210 nm (retention time 5 min). These measurements were carried out as a check for partitioning.

3. Results and discussion

3.1. Biocatalysis

Our transconjugant strain *P. putida* MC2 was tolerant to octanol. This tolerance is exceptional and essential for the kind of bioconversions that are described here. In contrast, Harrop et al. (1992) showed that neither free nor immobilized *P. putida* UV4 cells could resist the presence of a second phase of octanol. Furthermore, *P. putida* MC2 does not need antibiotics to maintain stable integration of the pertinent genes and to ensure stable 3-methylcatechol production from toluene (Hüsken et al., 2001a); it is thus a good candidate for two-phase 3-methylcatechol production.

3.2. Single-phase fermentations

As a reference for two-phase experiments, growth and 3-methylcatechol production in single-phase LB medium by *P. putida* MC2 in a bioreactor were monitored (Fig. 1). Under the conditions applied, the production rate was highest at late-exponential growth. Once growth stopped at 5 mM of 3-methylcatechol ($t = 7$ h),

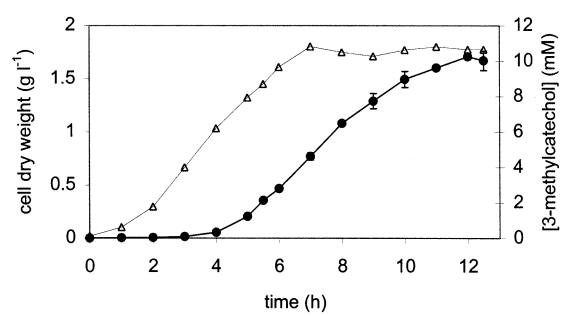

Fig. 1. Typical graph of growth of *Pseudomonas putida* MC2 (Δ) and accumulation of 3-methylcatechol (●) in a bioreactor with aqueous medium and a constant toluene concentration of 1.0 mM. Error bars indicate standard deviation between two samples.

production also ceased within a few hours ($t =$ 12 h) at a product concentration of 10 mM. The final cell-dry-weight concentration was 1.7 g l^{-1}; without toluene, and thus without 3-methylcatechol production, a biomass concentration of 2.1 g l^{-1} was found. On minimal growth media with glucose or succinate, this difference was more pronounced (Hüsken et al., 2001b). The nearly constant toluene concentration of 1.0 mM does not actually affect the growth rate (results not shown). On the other hand, on succinate medium 0.7 mM of 3-methylcatechol decreased the maximum specific growth rate with 50% (Hüsken et al., 2001a), while above 6 mM, no growth was observed at all, although cells were still active. This agrees with the results of Fig. 1. Elevated product concentrations thus inhibit growth and consequently production in single-phase 3-methylcatechol production processes.

3.3. Partitioning

P. putida MC2 is tolerant to 1-octanol and active in its presence. Also, octanol hardly dissolves in water, while partitioning is good: both substrate and product dissolve much better in octanol than in water. From the calculated $\log P_{o/w}$ of 3-methylcatechol (1.58; Meylan and Howard, 1995), a partition coefficient of 38 would be expected for 3-methylcatechol over an octanol/water two-phase system. A somewhat lower partition coefficient of 22 ($\log P_{o/w} = 1.34$, based on mM) was found experimentally for 3-methylcatechol over octanol/LB medium (results not shown). Partitioning thus seemed appropriate and sufficient for improving the production process.

3.4. Small-scale two-phase optimization experiments

Various conditions for growth and 3-methylcatechol production in two-phase systems were examined in 250 ml Boston bottles containing 10 ml of total liquid. The octanol phase contained a large reservoir of toluene: 400 times the aqueous concentration, according to $\log P_{o/w} =$ 2.60 (Osborne et al., 1990). This was checked experimentally. Because of the high volatility of toluene, quantitative measurements of aqueous toluene concentrations by HPLC were unreliable. Therefore, from a bottle containing air, LB medium, octanol and dissolved toluene, the gas phase toluene concentration was analyzed by gas chromatography. Calculations using this measured toluene concentration and the water–air distribution ratio (Amoore and Hautala, 1983) revealed that the aqueous toluene concentration in LB medium was 1 mM, as expected, assuming toluene partitioning between aqueous LB medium and air is equal to partitioning between water and air (3.8:1). Using this same assumption, the toluene mass balance then ascertains that 400 mM of toluene is present in octanol and the $\log P_{o/w}$ is thus 2.60.

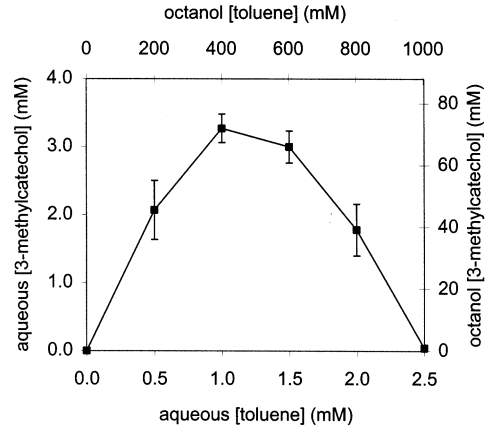

Fig. 2. Accumulated aqueous and octanol concentrations of 3-methylcatechol as a function of aqueous and octanol toluene concentration in a 1:1 octanol/aqueous two-phase system (small scale).

One-fourth or less of the total toluene quantity was converted, resulting in an aqueous toluene concentration that always remained higher than 75% of its initial value. To obtain the optimal toluene concentration for 3-methylcatechol production, bottles with 1:1 organic/aqueous two-phase systems were tested. Highest product concentrations were obtained with a starting aqueous toluene concentration of 1.0 mM (Fig. 2); this value was chosen for the subsequent bioreactor experiments.

Table 1
Batch 3-methylcatechol (3-MC) accumulation (mM and mmol)[a]

Phase ratio (octanol over LB, v/v)	10 ml scale, shake flasks		0.80 l scale, bioreactor	
	[3-MC] (mM) oct:LB[b]	3-MC_{total} (mmol)[c]	[3-MC] (mM) oct:LB[b]	3-MC_{total} (mmol)[c]
0:10	7.8 (±0.64)	0.078 [0]	9.5 (±1.9)	8 [0]
2:8	83:3.8 (±0.24)	0.20 [0.17]	65:3.0 (±0.35)	12 [10]
4:6	74:3.4 (±0.09)	0.32 [0.30]	54:2.5 (±0.25)	19 [17]
5:5	72:3.3 (±0.21)	0.38 [0.36]	48:2.2 (±0.28)	20 [19]
8:2	24:1.1 (±0.13)	0.20 [0.19]	ND[d]	ND[d]

[a] Standard deviations over aqueous (LB) concentrations are shown in brackets. All incubations contained 1.0 mM of toluene in the water phase at time $t = 0$; in small-scale experiments, this concentration dropped during production, while in 0.80 l experiments, the LB toluene concentration was kept constant by aeration with air that contained toluene.
[b] Octanol product concentration (mM):LB product concentration (mM).
[c] In square brackets the amount of 3-methylcatechol in octanol (mmol).
[d] ND, not determined.

Table 1 shows final product concentrations from small-scale experiments at various organic/aqueous phase ratios between 0 and 80 vol.% of octanol. The highest product concentration in octanol was observed at 20 vol.% of octanol, while the highest overall product accumulation was measured at an octanol fraction of 50 vol.% (72 mM of product in octanol, corresponding to an overall concentration of 38 mM; Table 1). In bioreactor experiments, various phase ratios were tested as well (see below).

In aqueous systems, the best medium for high specific production rates was minimal glucose medium (Hüsken et al., 2001b), but in two-phase Boston bottles this medium showed a long lag time of 40 h (results not shown). Final product concentrations in systems with minimal glucose and with LB medium were comparable. Therefore, LB medium was used in two-phase studies. It was assumed that cells had sufficient growth and maintenance substrate when using LB medium: four-fold concentrated LB medium did not improve production of 3-methylcatechol. In Boston bottles, the pH was not controlled. Initially, it was 7, but it increased to 8.5 upon growth of *P. putida* MC2. At higher pH, the product is less stable, resulting in a brown medium color at the end of the incubation due to e.g. polymerization. This implies that more product was accumulated than was measured.

3.5. Organic/aqueous two-phase fermentations

Small-scale experiments were scaled up to 0.80 l experiments in a bioreactor, with the advantages of pH control, constant toluene concentrations and easier sampling. The bioreactor contained 0.80 l of liquid at varying organic/aqueous phase ratios. Table 1 shows results of bioreactor experiments. Overall production was increased as compared to single-phase aqueous medium and high 3-methylcatechol concentrations were obtained in octanol.

Fig. 3 shows experiments in which 3-methylcatechol concentrations vs. time were measured in a

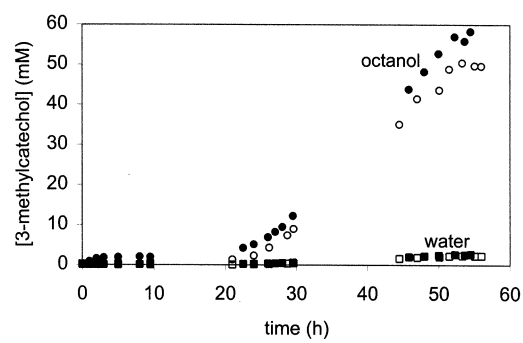

Fig. 3. Accumulation of 3-methylcatechol in organic/aqueous two-phase system. In the bioreactor, 40 vol.% of octanol was present. Results of two independent experiments are depicted. ●, ○, 3-methylcatechol concentration in octanol phase; ■, □, concentration in aqueous phase.

system with 40 vol.% of octanol. In these fermentations, an overall product concentration of 23 mM was obtained, consisting of 54 mM in the octanol phase and 2.5 mM in the aqueous phase. CO_2 production decreased after 50 h (results not shown), indicating that cells were no longer active. Octanol kept substrate and product concentrations low during the reaction and volumetric production was clearly improved.

The time to attain the final product concentration (53 h, Fig. 3) was longer than in single-phase systems (12 h, Fig. 1), because cells needed to adapt to the organic solvent.

Again, total product accumulation was highest at 50 vol.% of octanol. In the octanol phase, 48 mM of 3-methylcatechol was present, vs. 2.2 mM in aqueous medium, corresponding to a volume-averaged concentration of 25 mM. Highest concentrations in octanol were achieved at the lowest phase ratio (2:8), where 65 mM of 3-methylcatechol was present in octanol (Table 1).

It is believed that differences between final product concentrations in small-scale and in 0.8 l bioreactor experiments are a result of upscaling. Only when no octanol was present, bioreactor experiments showed higher 3-methylcatechol concentrations than small-scale experiments. This was caused by toluene limitation in single-phase small-scale experiments, whereas in the bioreactor, the toluene concentration was kept constant. In the small-scale incubations, flasks were shaken, while in the 0.8 l bioreactor, culture medium and octanol were stirred and aerated. This resulted in a lower activity, probably due to more stress to the cells, as was found previously (Harrop et al., 1992). At the end of the process, a stationary state was reached, indicating that changes in overall product concentrations at different scales were not caused by slow product transfer to the octanol phase or by a possibly lower area available for this transfer at larger scale.

The colour of the emulsion changed. At the start, it was off-white, subsequently gray, and via brownish red it changed to dark brown at the end of the incubation, probably as a result of product polymerization (Shirai, 1987; Robinson et al., 1992). In a buffer at pH 7, 9% of 3-methylcatechol disappears in 24 h (Hüsken et al., 2001a). It is therefore assumed that the actually produced amount of 3-methylcatechol was higher than the measured amount. Product stability was higher at a lower pH, but then cells had more severe problems in coping with the organic phase.

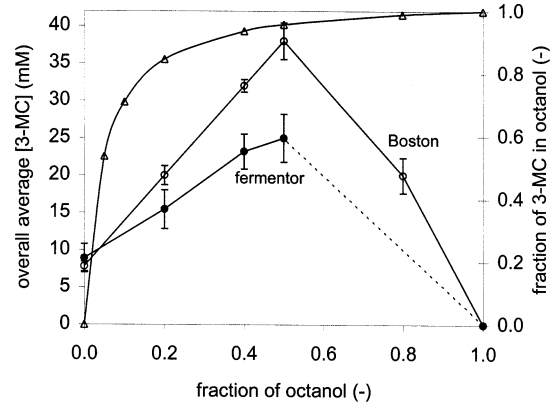

Fig. 4. Average product concentration over total volume as a function of phase ratio octanol/water, both in small-scale Boston bottles (○) and in bioreactor experiments (●). Error bars indicate standard deviations. (△) Fraction of product in organic phase as a function of phase ratio octanol/water.

3.6. Optimization of phase ratio

Figure 4 shows averaged product concentrations and the fraction of the product that is present in the octanol phase, as a function of the volume fraction of octanol phase. Linear plots should be expected for averaged 3-methylcatechol concentrations when production would stop at a constant inhibitory product concentration in the aqueous phase. From Table 1 however, it is clear that the final aqueous concentrations were not constant, but decreased at increasing octanol hold-ups. If the single-phase aqueous concentration of 10 mM would have been achieved in all two-phase incubations, Fig. 4 would show steeper, linear plots of the overall 3-methylcatechol concentration. Still, overall concentrations were much higher than in single-phase systems due to the large octanol reservoir, satisfying our requirements.

Optimal production of 3-methylcatechol is defined by the octanol fraction at which: (1) a

high overall 3-methylcatechol concentration is achieved; (2) the fraction of product present in the octanol phase is high; and (3) the total process time to produce the acquired amount of product is low. The fraction of product present in octanol, for downstream processing, does not change much above 40 vol.% of octanol (Fig. 4). It is therefore reasonable to regard an octanol hold up of approximately 50% as optimal for the first two requirements: accumulating large quantities of 3-methylcatechol and achieving a high product recovery in octanol.

Process times (including lag time) were not taken into account in Fig. 4. In small-scale experiments, process times were rather similar for different volume fractions of octanol, except for 80 vol.%, were the process time was doubled. In bioreactor experiments however, larger differences were found: the higher the octanol fraction, the higher the process time. It is thus crucial to define the importance of short process times (low octanol volume fraction) and high production and product recovery (50 vol.% of octanol).

For the recovery of 3-methylcatechol from octanol, the octanol phase can be extracted with 1 M NaOH. The sodium salt of 3-methylcatechol will then be present in the aqueous phase. The catechol can be extracted in a different organic phase, that can be evaporated to yield 3-methylcatechol.

In conclusion, without the need to add antibiotics, an improved set-up for high production levels of 3-methylcatechol was achieved, using a stable solvent-tolerant bacterium in an organic/aqueous two-phase system. This integrated process increased overall product accumulation and entailed a first extractive step in downstream processing.

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References

Amoore, J.E., Hautala, E., 1983. Odor as an aid to chemical safety: odor thresholds compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. J. Appl. Toxicol. 3, 272–290.

Arnow, L.E., 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine–tyrosine mixtures. J. Biol. Chem. 118, 531–537.

Bont, J.A.M. de, 1998. Solvent-tolerant bacteria in biocatalysis. Trends Biotechnol. 12, 493–499.

Brink, L.E.S., Tramper, J., 1985. Optimization of organic solvent in multiphase biocatalysis. Biotechnol. Bioeng. 12, 1258–1269.

Cesário, M.T., Turtoi, M., Sewalt, S.F.M., Beeftink, H.H., Tramper, J., 1996. Enhancement of the gas-to-water ethene transfer coefficient by a dispersed water–immiscible solvent: effect of the cells. Appl. Microbiol. Biotechnol. 46, 497–502.

Cesário, M.T., Beverloo, W.A., Tramper, J., Beeftink, H.H., 1997. Enhancement of gas–liquid mass transfer rate of apolar pollutants in the biological waste-gas treatment by a dispersed organic solvent. Enzyme Microb. Technol. 21, 578–588.

Collins, A.M., Woodley, J.M., Liddell, J.M., 1995. Determination of reactor operation for the microbial hydroxylation of toluene in a two-liquid phase process. J. Ind. Microbiol. 14, 382–388.

Collins, L.D., Daugulis, A.J., 1999a. Benzene/toluene/*p*-xylene degradation. Part I. Solvent selection and toluene degradation in a two-phase partitioning bioreactor. Appl. Microbiol. Biotechnol. 52, 354–359.

Collins, L.D., Daugulis, A.J., 1999b. Benzene/toluene/*p*-xylene degradation. Part II. Effect of substrate interactions and feeding strategies in toluene/benzene and toluene/*p*-xylene fermentations in a partitioning bioreactor. Appl. Microbiol. Biotechnol. 52, 360–365.

Cruden, D.L., Wolfram, J.H., Rogers, R.D., Gibson, D.T., 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic–aqueous) medium. Appl. Environ. Microbiol. 58, 2723–2729.

Ennis, M.D., Ghazal, N.B., 1992. The synthesis of (+)- and (−)-Flesinoxan: application of enzymatic resolution methodology. Tetrahedron Lett. 33, 6287–6290.

Harbron, S., Smith, B.W., Lilly, M.D., 1986. Two-liquid phase biocatalysis: epoxidation of 1,7-octadiene by *Pseudomonas putida*. Enzyme Microb. Technol. 8, 85–88.

Harrop, A.J., Woodley, J.M., Lilly, M.D., 1992. Production of naphtalene-*cis*-glycol by *Pseudomonas putida* in the presence of organic solvents. Enzyme Microb. Technol. 14, 725–730.

Hartog, J., Wouters, W., 1988. Flesinoxan hydrochloride. Drugs of the future 13, 31–33.

Held, M., Schmid, A., Kohler, H.-P.E., Suske, W., Witholt, B., Wubbolts, M.G., 1999. An integrated process for the production of toxic catechols from toxic phenols based on a designer biocatalyst. Biotechnol. Bioeng. 62, 641–648.

Hüsken, L.E., Beeftink, H.H., Bont, J.A.M. de, Wery, J., 2001a. High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. Appl. Micrbiol. Biotechnol., DOI 10.1007/s002530000566..

Hüsken, L.E., Bont, J.A.M. de, Beeftink, H.H., Tramper, J., Wery, J., 2001b. Optimisation of microbial 3-methylcatechol production as affected by culture conditions, Submitted.

Isken, S., Derks, A., Wolffs, P.F.G., Bont, J.A.M. de, 1999. Effect of organic solvents on the yield of solvent-tolerant *Pseudomonas putida* S12. Appl. Environ. Microbiol. 65, 2631–2635.

Lilly, M.D., Woodley, J.M., 1996. A structured approach to design and operation of biotransformation processes. J. Ind. Microbiol. 17, 24–29.

Marshall, C.T., Woodley, J.M., 1995. Process synthesis for multi-step microbial conversions. Bio/Technology 13, 1072–1078.

Meylan, W.M., Howard, P.H., 1995. Atom/fragment contribution method for estimating octanol–water partition coefficients. J. Pharm. Sci. 84, 83–92.

Osborne, S.J., Leaver, J., Turner, M.K., Dunnill, P., 1990. Correlation of biocatalytic activity in an organic–aqueous two-liquid phase system with solvent concentration in the cell membrane. Enzyme Microb. Technol. 12, 281–291.

Rekker, R.F., de Kort, M., 1979. The hydrophobic fragmental constant; an extension to a 1000 data point set. Eur. J. Med. Chem. — Chimica Therapeutica 14, 479–488.

Robinson, G.K., Stephens, G.M., Dalton, H., Geary, P.J., 1992. The production of catechols from benzene and toluene by *Pseudomonas putida* in glucose fed-batch culture. Biocatalysis 6, 81–100.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual, 2nd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scharrenburg, G.J.M. van, Frankena, J., 1996. Biokatalyse helpt farmaceutische industrie bij asymmetrische synthese. Chemisch Magazine, 284–286.

Shirai, K., 1987. Catechol production from benzene through reaction with resting and immobilized cells of a mutant strain of *Pseudomonas*. Agric. Biol. Chem. 51, 121–128.

Tramper, J., Vermuë, M.H., Beeftink, H.H., Stockar, U. von (Eds.), 1992. Progress in Biotechnology 8. Biocatalysis in Non-Conventional Media. Elsevier Science Publishers B.V, Amsterdam, The Netherlands.

Vermuë, M., Sikkema, J., Verheul, A., Bakker, R., Tramper, J., 1993. Toxicity of homologous series of organic solvents for the Gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnol. Bioeng. 42, 747–758.

Weber, F.J., Bont, J.A.M. de, 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim. Biophys. Acta 1286, 225–245.

Wery, J., Mendes da Silva, D.I., Bont, J.A.M. de, 2000. A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemical. Appl. Microbiol. Biotechnol. 54, 180–185.