PRODUCTION OF CATECHOLS

microbiology and technology

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PRODUCTION OF CATECHOLS

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Contents

Chapter 1

Introduction

In the past decades, many breakthroughs were established in the field of biosciences. Many biological facts were resolved and the new insights in the many bioscientific disciplines (genetics, microbiology, biochemistry, process engineering et cetera) were integrated. In the area of biocatalysis, knowledge in the scientific disciplines was combined and applied to accomplish specific biological conversions. Micro-organisms are capable of carrying out many enzymatic reactions, that can be highly selective and/or specific. Some may be used for industrial purposes, because of economic and environmental considerations.

BIOSCIENCES AND GREEN CHEMISTRY

For industrial bioproduction of a compound, different processes may be available that can be based either on whole cells or on cell-free systems. Whole cells are used if a number of subsequent reactions take place that lead to a product. Examples are:

- the production of 5-methylpyrazine-2-carboxylic acid at Lonza, by the selective oxidation of a methyl substituent on an aromatic *N*-heterocycle (pyrazine), by the xylene oxidation pathway of *Pseudomonas putida* ATCC 33015 (Schmid *et al.*, 2001);
- production of L-aspartic acid, an aspartame (sweetener) precursor, via enzymes that are expressed in *Escherichia coli* (Orum and Rasmussen, 1992), on a thousand tons-per-annum scale at DSM (Schmid *et al.*, 2001);
- production of L-lysine by *Bacillus laterosporus*, for example on millet (carbon source) and soy bean (nitrogen source) (Umerie *et al.*, 2000);

- L-lysine production from glucose using *C. glutamicum* (Marx *et al.*, 1997);
- the temperature-induced production of the amino acid L-glutamate from glucose by a temperature-sensitive strain of *Corynebacterium glutamicum* (Delaunay *et al.*, 1999);
- L-isoleucine production by *C. glutamicum* (Eggeling *et al.*, 1997).

Likewise, different isolated enzymes are used in many processes such as:

- lipase, in the production of Diltiazem – a cardiovascular drug (Schulze and Wubbolts, 1999), in the production of antifungal agents by Schering-Plough (Morgan *et al.*, 1997), and for the production of (*1S*,*2S*)-trans-2-methoxycyclohexanol at GlaxoSmithKline (Stead *et al.*, 1996; Schulze and Wubbolts, 1999);
- protease, in the production of an aspartame precursor (Hanzawa, 1999; Schulze and Wubbolts, 1999);
- thermolysine, in the production of aspartame, by the Holland Sweetener Company, a joint venture of Tosoh and DSM (Schmid *et al.*, 2001);
- immobilised penicillin G acylase, in the production of cephalosporins at DSM (Bruggink, 2001).

Cofactor-dependent enzymatic reactions usually are carried out in whole cells in order to allow a cheap regeneration of the cofactor. These reactions can be seen as positioned in between whole cell bioprocesses that usually are referred to as fermentations, and bioprocesses that are based on isolated enzymes. Many examples are available, demonstrating the potential of these cofactor-dependent whole-cell biocatalytic reactions. The examples cited are processes that either have replaced conventional chemical processes or that have opened completely new options in producing a specific chemical (Table 1). Bruggink (2001) for instance, describes how bioproduction of semi-synthetic antibiotics has replaced conventional chemical production.

Biological pathways that are more specific, more efficient and, additionally, less polluting for the environment than chemical processes are referred to as “green”. Benefits may include a higher product yield, a lower energy requirement, milder reaction conditions and less by-products or waste (Held *et al.*, 1998; Miller and Peretti, 1999; Van Scharrenburg and Frankena, 1996).

Table 1 Examples of commercial microbial production processes. The companies that carry out a specific reaction are written in brackets.

Biocatalyst	Process	Reference
Penicillium chrysogenum	production of adipyl-7-ADCA (DSM)	Bruggink, 2001
recombinant *Bacillus subtilis*	production of riboflavin (vitamin B_2) (Hoffmann-La Roche)	Petersen and Kiener, 1999
Anthrobacter oxydans NRRL-B-3603 hydroxylase	production of 6-hydroxy-S-nicotine (Lonza)	Schmid *et al*., 2001
P. putida ATCC 33015, xylene oxidation pathway	production of 5-methylpyrazine-2-carboxylic acid (Lonza)	Schmid *et al*., 2001
Pseudomonas putida hydratase	production of 5-cyanopentanamide (DuPont)	Stieglitz *et al*., 1996; Schulze and Wubbolts, 1999
Pseudomonas halo acid dehalogenase	resolution of (S)-2-chloropropionic acid (production of herbicides) (Astra Zeneca)	Taylor, 1997; Schulze and Wubbolts, 1999
Agrobacterium HK1349 hydratase	production of L-carnitine (Lonza)	Zimmerman *et al*., 1995; Schulze and Wubbolts, 1999

There is an increasing demand from society for green processes, to sustain our natural environment (Held *et al.*, 2000; Miller and Peretti, 1999; Schmid *et al.*, 2001). Anastas and Warner (1998) have composed twelve principles of green chemistry (Table 2), which might be used as a consideration in the design of a green process. These principles apply for processes in general, either for chemical or biological processes. Strong points for bioprocesses inherently are prevention of waste (specific and selective production routes), atom economy (high product yield) and energy efficiency (low energy requirement and mild reaction conditions) (Held *et al.*, 1998; Tramper *et al.*, 2001).

Table 2 Twelve principles of green chemistry, according to Anastas and Warner (1998).

		12 principles of green chemistry	
1	prevention	✓	prevent waste rather than treating it or cleaning it up after it has been created
2	atom economy	✓	maximise yield of product on substrate(s)
3	less hazardous chemical syntheses	✓	use and generate non-toxic compounds, that are not hazardous to the environment
4	designing safer chemicals	✓	design products to effect their desired function, while minimising their toxicity
5	safer solvents and auxiliaries	✓	use of auxiliary substances should be circumvented, or innocuous when used
6	design for energy efficiency	✓	minimise energy requirements (environmental and economic impact) and when possible, operate at ambient temperature and pressure
7	use of renewable feed stocks	✓	raw material should be renewable, rather than depleting, whenever technically and economically practicable
8	reduce derivatives	✓	unnecessary derivatisation should be minimised or avoided, to prevent additional waste streams and the use of additional reagents
9	catalysis	✓	catalytic reagents – as selective as possible – are superior to stoichiometric reagents
10	design for degradation	✓	design products so that at the end of their function, they break down into innocuous compounds and don't persist in the environment
11	real-time analysis for pollution prevention	✓	methodologies have to be developed, to allow for real-time, in-process monitoring and control, prior to formation of hazardous substances
12	inherently safer chemistry for accident prevention	✓	minimise process potential for chemical accidents, including releases, explosions, and fire

BIOCATALYSIS

For the reasons as outlined above, growing numbers of scientists that have a background in any of the life sciences will be employed by chemical industries. Biocatalysis, as a means to accomplish new and difficult conversions, also finds its way to organic chemists and its use for industrial synthetic chemistry is on the verge of significant growth (Schmid *et al.*, 2001).

To develop a biocatalytic process, the contribution of many different experts is required. In Figure 1, a general overview is given of expertises involved. The aim of a biocatalytic production process is to convert a suitable substrate into the desired product. A biocatalyst must be found or constructed, a process has to be set up, and the resulting overall conversion has to be economically feasible (Schmid *et al.*, 2001). In order to achieve this, the whole cycle in Figure 1 has to be followed.

A key element in biocatalysis, central in Figure 1, is toxicity of substrate or product towards the biocatalyst. For the design and optimisation of a biological process, this is a crucial aspect to consider.

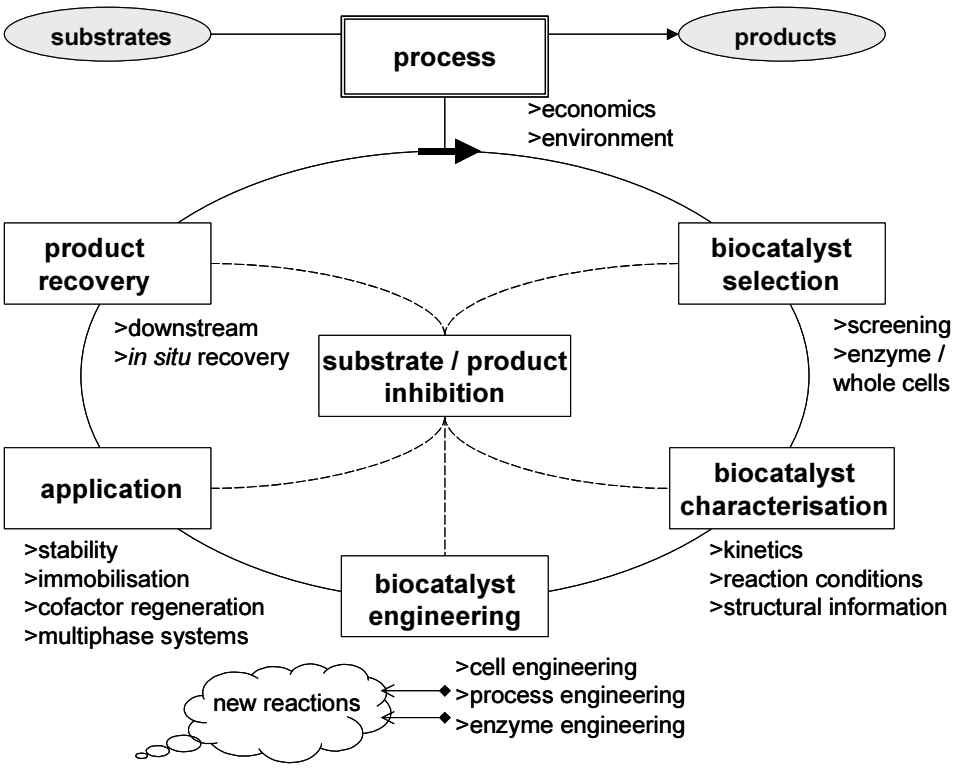

Figure 1 The development of a biocatalytic process: the biocatalysis cycle (adapted from Schmid *et al.*, 2001).

Product accumulation and biocatalyst inhibition

In many biological production processes, high substrate concentrations, as well as the extended accumulation of product, are inhibitory or toxic to the enzymatic or cellular biocatalyst (De Bont, 1998; Collins *et al.*, 1995; Hack *et al.*, 2000; Harrop *et al.*, 1992; Marshall and Woodley, 1995). Bioproduction of for instance ethanol is limited by ethanol toxicity for the micro-organism. High product concentrations however are attractive from the point of view of downstream processing. In the case of a toxic product, production stops when the product concentration increases above a certain level. The same considerations apply for toxic substrates. However, a toxic substrate or product does not rule out a biocatalytic process.

Dealing with substrate and product toxicity

Many solutions to alter and improve process conditions with respect to inhibitory phenomena are based on the maintenance of a low toxic compound(s) concentration (Figure 2). Here we make no distinction between inhibition (reversible) and toxicity (irreversible), since the ways to cope with these problems are similar. The product concentration in the environment of the biocatalyst can be kept low by *in situ* product removal, e.g. by adsorption onto some solid phase (Held *et al.*, 1998; Lilly and Woodley, 1996; Robinson *et al.*, 1992), by extraction into a second aqueous phase in aqueous two-phase systems (Van Berlo *et al.*, 1998; Den Hollander *et al.*, 1998; Rogers *et al.*, 1998; Walter *et al.*, 1985), or by extraction into an organic solvent phase (Choi *et al.*, 1999; Collins and Daugulis, 1999; Harbron *et al.*, 1986; Westgate *et al.*, 1998). In some cases, it may be practical to have the product undergo an extra follow-up reaction (Figure 2), to prevent it from damaging the biocatalyst, or rather not to proceed with the last step in a production process, but to end with a non-toxic precursor. This precursor might consecutively be converted chemically to obtain the product after separation from the biocatalyst (Robinson *et al.*, 1992).

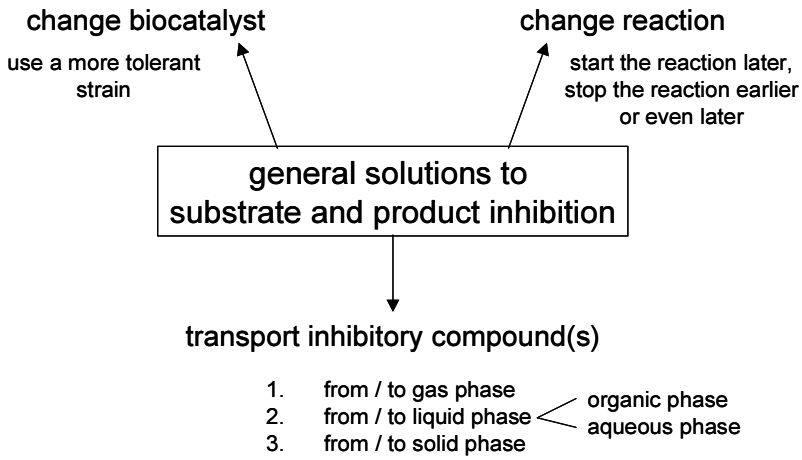

Figure 2 Some general solutions to substrate and product inhibition of a whole-cell biocatalyst.

As discussed before, an interaction between the different expertise areas (or disciplines) is needed for an efficient bioproduction process (Figure 1). Apart from the solutions offered by process engineering, some biological solutions are possible (Figure 1). For example, if a biocatalyst is solvent-tolerant (or can be developed as such), an organic/aqueous multi-phase process (Figure 2) becomes a possibility (De Bont, 1998). In their review on the search for the ideal biocatalyst, Burton *et al.* (2002) provide examples and available technologies for enhancing biocatalyst characteristics.

Now that the general picture of biocatalysis, problems and possible solutions is clear, we will focus on the biocatalytic production of a special class of products: catechols.

CATECHOLS

For the production of a large number of fine-chemicals, antibiotics, and flavour compounds, precursors are essential: they are the starting material for the production of the desired compound. Substituted catechols are relevant in this

respect (Figure 3). They serve as precursor in the fine-chemical industry and can be used in pharmaceutical production processes for the production of anti-hypertensive drugs (Ennis and Ghazal, 1992; Hartog and Wouters, 1988; Van Scharrenburg and Frankena, 1996). Also, catechols and their derivatives are important chemicals in the manufacture of synthetic flavours such as vanillin (Shirai, 1986). They are used as a reagent for photography, dyeing fur, rubber and plastic production (Schweigert *et al.*, 2001).

Among the catechols in general, catechols that have a substituent on the 3-position (Figure 3) are of particular interest. 3-Fluorocatechol (R = F in Figure 3) is a potential precursor in the synthesis of a wide range of pharmaceuticals, such as adrenergic catecholamines and biogenic amines (Kirk and Creveling, 1984). For the production of some antihypertensive pharmaceuticals, 3-nitrocatechol (R = NO_2 in Figure 3) is essential as a building block (Ennis and Ghazal, 1992; Kieboom *et al.*, 2001; Van Scharrenburg and Frankena, 1996).

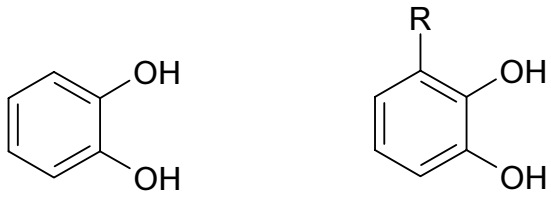

Figure 3 Catechol and 3-substituted catechol.

Catechol production

Chemical synthesis of 3-substituted catechols is difficult, due to low region-selectivity and low stability of the product (the product is oxidised faster than the substrate). In addition, stereo-selectivity in chemical synthesis of these catechols is inadequate. Also, many reaction steps are needed and consequently the recovery of chemically synthesised catechols on an industrial scale is rather poor (Van Scharrenburg and Frankena, 1996).

Biocatalysis provides alternative catechol production processes. While in chemical production, mixtures of 3- and 4-substituted catechols are obtained (Ennis and Ghazal, 1992), some micro-organisms are able to specifically produce 3-substituted catechols (Haigler and Spain, 1991; Robinson *et al.*, 1992; Zylstra *et al.*, 1988). Catechols can be produced from 2-substituted phenols by a monooxygenase from *Pseudomonas azelaica* HBP 1 (Held *et al.*, 1998). In another biocatalytic process, wild-type *Pseudomonas putida* F1 degrades toluene in two steps via *cis*-toluene dihydrodiol (toluene *cis*-glycol) and 3-methylcatechol by the sequential action of toluene dioxygenase and a dehydrogenase (Figure 4). Other enzymes then metabolise 3-methylcatechol, which therefore is not accumulated by this bacterium (Zylstra *et al.*, 1988). The efficiency and selectivity with which these biological reactions (Figure 4) can be conducted is unmatched in traditional chemistry (Miller and Peretti, 1999).

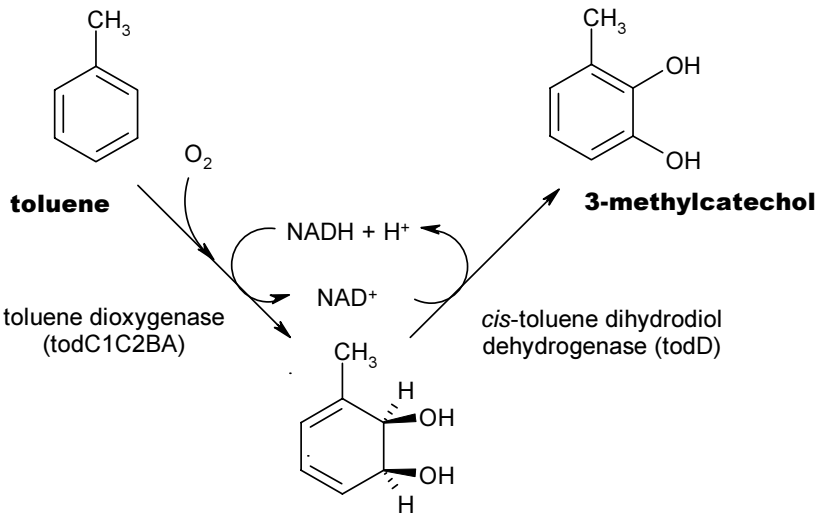

Figure 4 Microbial production of 3-methylcatechol in a two-step reaction from toluene via toluene *cis*-glycol, with a dioxygenase and a dehydrogenase from *Pseudomonas putida* (Zylstra *et al.*, 1988).

Catechol toxicity

Catechols are toxic towards many micro-organisms. High concentrations of 3-substituted catechols cause uncoupling of NADH conversion and product formation, leading to the formation of hydrogen peroxide (Suske *et al.*, 1997; Held *et al.*, 1998). 3-Methylcatechol inhibits substrate (toluene) oxidation (Robinson *et al.*, 1992). Many reactions can occur with biomolecules such as DNA, proteins and membranes, ultimately leading to irreversible damage, as a consequence of the chemical properties and reactions of catechols (Schweigert *et al.*, 2000, 2001). Therefore, tailor-made solutions are needed to biologically produce catechols. To ensure high concentrations and long-term bioproduction, negative effects on the micro-organism must be minimised. This can be achieved in different ways (Figure 2). Catechols are not very stable under physiological conditions, which limits the process. The guidelines that are specified in Figure 1 are of major importance in choosing and building the process.

In Table 3, examples of catechol production processes are depicted, in which high concentrations of substrate or product caused severe problems for the biocatalyst, and it is shown how the authors dealt with these problems.

AIM

The aim of this research project was to investigate and to develop a microbial process for optimal production of 3-substituted catechols, in terms of product concentrations and production rates. In the background of minimising the toxic effects, we chose not to study adsorption, that was already extensively studied (Table 3), but to examine the use of a second, organic phase in the process. Depending on costs of the reactor (high production rate required) or costs of downstream processing of the product (high product concentration required), the decisive process optimisation parameter can be attained. There are two ways to optimise catechol production: altering the micro-organism or changing the process conditions. We did both.

Table 3 Examples of bioprocesses in which glycols and catechols (Figure 4) caused problems. In most of the below cases, toxic substrates were fed below their inhibitory level to diminish loss of activity.

Biocatalyst	Process	Constraints	Solution	Reference
Escherichia coli JM101	production of 3-substituted catechols	substrate (phenol) and product toxicity	product adsorption	Held *et al*., 1998
Escherichia coli JM101 (pHBP461)	production of 3-phenylcatechol	substrate (2-phenylphenol) and product toxicity	product adsorption	Held *et al*., 1999
Pseudomonas putida UV4	production of toluene cis-glycol	substrate (toluene) toxicity	organic solvent extraction	Lilly and Woodley, 1996
Pseudomonas putida ML2	production of 3-fluorocatechol	product toxicity	product adsorption	Lilly and Woodley, 1996; Lynch *et al*., 1997
Pseudomonas putida 2313	production of 3-methylcatechol	product toxicity	product adsorption	Robinson *et al*., 1992
Pseudomonas putida 6(12)	production of catechol	product toxicity	product adsorption	Robinson *et al*., 1992

BIOCATALYST AND CATECHOL PRODUCTION

In this project, the conversion of toluene to 3-methylcatechol was studied as a model reaction. For this purpose, a new *Pseudomonas putida* strain was constructed, that was 1) over-expressing the genes responsible for catechol production, 2) incapable of enzymatically degrading the desired product, and 3) solvent tolerant.

This model biocatalyst, designated *Pseudomonas putida* MC2, was tested for the production of 3-substituted catechols. A main advantage of this process

is the regeneration of cofactor NADH (Figure 1, Figure 4) by the biocatalyst. For this pathway (Figure 4), especially lines 1, 2, 6, 8 and 9 from Table 2 apply, indicating the green potential of this catechol bioproduction process.

Catechol production by *P. putida* MC2 and thesis outline

In Chapter 2, the construction of the novel strain *P. putida* MC2 is described. We found that growth of *P. putida* MC2 was needed in the optimal production process. Chapter 3 illustrates the influence of different culture conditions on growth and bioproduction of 3-methylcatechol by this strain in aqueous media. As stated above however, product accumulation in the aqueous medium may severely limit the biocatalytic activity when the product is inhibitory or toxic. This was also found to be the case in our process. A high concentration of the product 3-methylcatechol (or the substrate toluene) in the culture medium resulted in cell death and the inevitable end of the production process. To overcome this problem, a different strategy was chosen in Chapter 4.

To increase production, the product was removed from the production medium during the process by introducing a second organic phase, into which the product is extracted from the aqueous production medium (Figure 2), therewith preventing exposure of the biocatalyst to high concentrations of 3-methylcatechol. Some micro-organisms are tolerant to organic solvents (Inoue and Horikoshi, 1989; Isken and De Bont, 1998; Ramos *et al.*, 1998). Since this solvent tolerance was especially found in *Pseudomonas* strains, we tested the survival and activity of our strain, *P. putida* MC2, in the presence of various solvents that normally would not allow bacterial growth, but that would extract the product efficiently. Amongst others, 1-octanol was tested and was found appropriate. Partitioning of both substrate and product over octanol and the aqueous culture medium was good and our strain survived and maintained activity, although some adaptation time was needed. This process set-up provided an integrated extractive step for the recovery of 3-methylcatechol (Figure 5). In Chapter 4, organic/aqueous two-phase bioproduction of 3-methylcatechol is described.

A similar biphasic process was also carried out with a membrane in-between the aqueous and octanol phases. This membrane prevented direct contact between the octanol phase and the bacteria in the aqueous phase. We expected a shorter adaptation time due to the reduction of octanol phase toxicity for the bacteria (Bar, 1988). Furthermore, downstream processing would be facilitated, since both phases are kept separate. Chapter 5 deals with this two-phase bioproduction of 3-methylcatechol in the presence of a hydrophobic membrane. Preliminary downstream processing experiments of the octanol phase are also discussed in this chapter.

In addition, a mathematical description of one- and two-phase growth and 3-methylcatechol production was accomplished in Chapter 6. This model facilitates rational process design to optimise the process with respect to the desired outcome. A general discussion follows in Chapter 7.

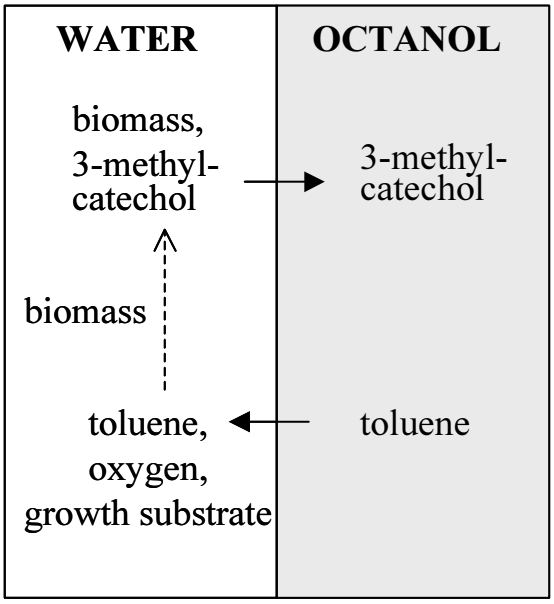

Figure 5 Schematic representation of a two-phase 3-methylcatechol bioproduction process.

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Chapter 2

High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system

ABSTRACT

The bioconversion of toluene into 3-methylcatechol was studied as a model system for the production of valuable 3-substituted catechols in general. For this purpose an improved microbial system for the production of 3-methylcatechol was obtained. *Pseudomonas putida* strains, that contain the *todC1C2BAD* genes involved in the conversion of toluene into 3-methylcatechol, were used as hosts for introduction of extra copies of these genes by means of a novel integrative expression system. A construct was made containing an expression cassette with the *todC1C2BAD* genes cloned under control of the inducible regulatory control region for naphthalene and phenanthrene degradation *nagR*. Introduction of this construct into wild type *P. putida* F1, which degrades toluene via 3-methylcatechol, or into mutant *P. putida* F107 that accumulates 3-methylcatechol, yielded biocatalysts that carried multiple copies of the expression cassette. As a result up to 14 mM (1.74 g l^{-1}) of 3-methylcatechol was accumulated and the specific production rate reached a level of 105 µmol min^{-1} g CDW^{-1}, which is four times higher compared to other catechol production systems. It was shown that these properties were stably maintained in the biocatalysts, without the need for antibiotics in the production process. This is an important step for obtaining designer biocatalysts.

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INTRODUCTION

Substituted catechols are valuable precursors in pharmaceutical production processes (Ennis and Ghazal, 1992; Hartog and Wouters, 1988; Van Scharrenburg and Frankena, 1996) and catechol and its derivatives are important chemicals used in manufacturing synthetic flavours such as vanillin (Shirai, 1986). Chemical synthesis of 3-substituted catechols is complex and several reaction steps are required, resulting in a low overall yield (Held *et al.*, 1998; Van Scharrenburg and Frankena, 1996). Therefore, alternatives for production of these compounds are being investigated.

Bioproduction of 3-substituted catechols is possible by conversion of aromatic hydrocarbons into catechols (Robinson *et al.*, 1992; Spain and Gibson, 1988; Warhurst *et al.*, 1994; Zeyer *et al.*, 1985). For this purpose, Held *et al.* have used a monooxygenase from *Pseudomonas azelaica* HBP 1 to produce catechols from 2-substituted phenols (Held *et al.*, 1998). The responsible *hbpA* gene for this monooxygenase was also expressed in *Escherichia coli* JM101 on a multi-copy plasmid, resulting in a 3- to 5-fold higher amount of the monooxygenase and preventing enzymatic degradation of the product (Held *et al.*, 1998). A drawback of plasmid-encoded production systems, however, is the need for antibiotics to maintain stable production. Alternatively, mutant strains have been used for the accumulation of catechols (Robinson *et al.*, 1992; Lilly and Woodley, 1996). The advantage of these mutants was the higher stability compared to recombinant strains that carry episomal plasmids. A disadvantage is the relatively low production rate. High productivity, however, is a prerequisite for a large-scale bioproduction of catechols that can economically compete with chemical synthesis. Recently, Panke *et al.* (1999) showed that chromosomal integration of genes under the *alk* regulatory system was a useful route for stable high-rate heterologous production of styrene oxide.

In the present study we address optimisation of 3-methylcatechol production from toluene as a model system. We have chosen to develop a stable homologous expression system tailored for *Pseudomonas putida*, because this genus is more tolerant to the detrimental effects of toxic fine

chemicals, like 3-methylcatechol (De Bont, 1998). Strains *P. putida* F1 and its 3-methylcatechol-accumulating mutant F107 were selected as hosts, because these strains already had the genes and machinery for optimal toluene conversion to 3-methylcatechol, increasing the chance of proper expression of a second set of the homologous genes. We describe the construction, genetic characterisation and properties of these strains, and the conditions needed for optimal production of 3-methylcatechol. In addition, the use of the expression system for production of other fine chemicals is discussed.

MATERIALS AND METHODS

DNA methods

Insert sequences were isolated from 0.7 % (w/v) agarose gels using the Geneclean II Kit (BIO 101 Inc.). DNA digestions and ligations were carried out using enzymes purchased from BRL and applied according to the suppliers recommendations. Total genomic DNA from *P. putida* strains was prepared by the CTAB procedure (Ausubel *et al.*, 1991). For DNA hybridisations, total DNA of different *P. putida* strains was digested, separated by agarose gel electrophoresis, and transferred to nylon filters according to standard protocols (Sambrook *et al.*, 1989). Hybridisations were done using the DIG DNA Labelling and Detection Kit, non-radioactive (Boehringer), according to the manufacturers recommendations. Sequencing of purified double-strand plasmid DNA was accomplished using AmpliTaq FS DNA fluorescent dye terminator reactions (Perkin-Elmer) in a Gene Amp PCR system 9600 (Perkin-Elmer). Sequencing products were detected using an Applied Biosystems 373A stretch automated DNA sequencer (Applied Biosystems Inc.). Nucleotide sequence analysis was carried out with the National Center for Biotechnology Information BLAST server (Altschul *et al.*, 1990).

Plasmid construction

Plasmid pTnJW4N was constructed in several steps (Figure 1). Initially, the *todC1C2BAD* genes were excised from plasmid pDTG602 (Zylstra and Gibson, 1989) on two DNA fragments by digesting with *Eco*RI/*Not*I and *Not*I/*Sal*I. These fragments were cloned simultaneously in plasmid pJW1NFL, cut with *Eco*RI and *Xho*I, downstream the regulatory control region for naphthalene and phenanthrene degradation from *Comamonas testosteroni* GZ42 (*nag*R/P*nag*Aa) (Wery and Zylstra, 1999), resulting in plasmid pJW2NFL. The *nag*R/P*nag*Aa-*todC1C2BAD* expression cassette was excised from pJW2NFL on two fragments by digesting with *Sst*I/*Bam*HI and *Bam*HI/*Xba*I. After the *Xba*I cohesive end was blunted with Klenow enzyme, the two fragments were cloned into the conjugatable suicide transposon donor TnMod-KmO (Dennis and Zylstra, 1998) cut with *Sst*I and *Swa*I, resulting in pTnJW4N.

Bacterial strains, media and growth of strains

P. putida MC1 and MC2 are 3-methylcatechol-overproducing strains derived from *P. putida* F1 (Zylstra *et al.*, 1988; Zylstra and Gibson, 1989) and F107 (Finette and Gibson, 1988), respectively, by transconjugation with pTnJW4N. *Escherichia coli* strain DH5α (*supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1) was used for transformation and amplification of recombinant plasmids according to standard methods (Sambrook *et al.*, 1989). LB broth (Sambrook *et al.*, 1989) was used as complete medium. Mineral salts medium was prepared according to Hartmans *et al.* (1989), with a 60 mM phosphate buffer at pH 7.0 and 80 mM of either succinate (MM/succ) or glucose (MM/gluc). Solid media contained 2 % (w/v) of agar. Ampicillin (50 μg/ml) and kanamycin (50 μg/ml) were added to maintain plasmids in *E. coli*. Kanamycin (50 μg/ml) was used in the selection for *P. putida* F1 and F107 TnJW4N mutants.

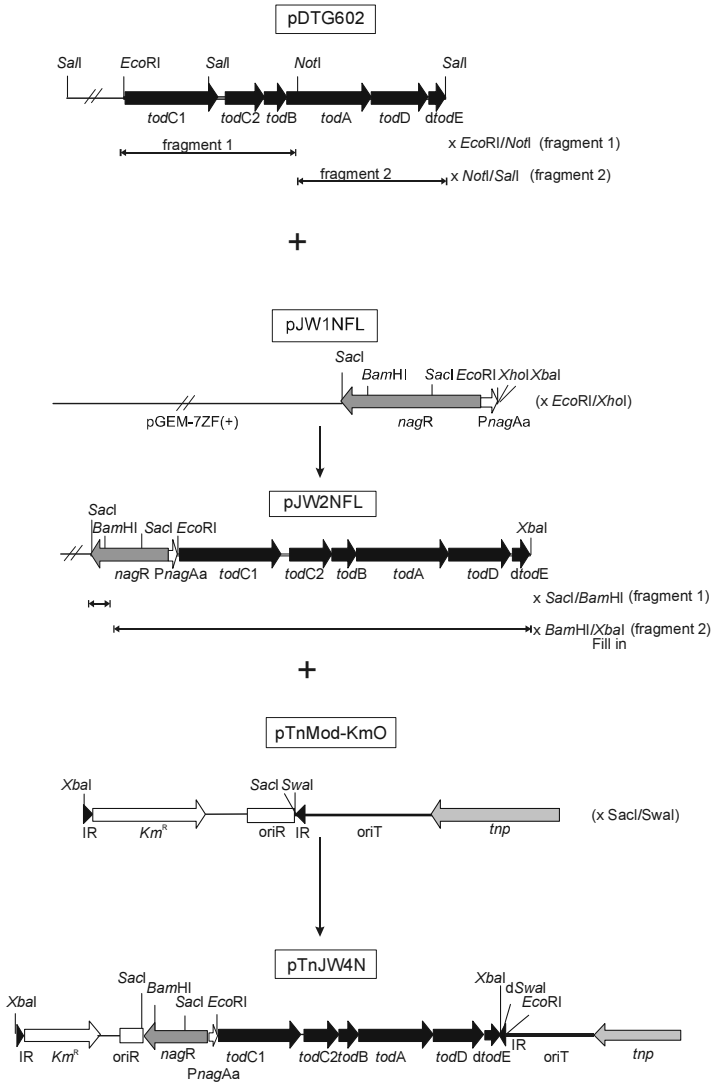

Figure 1 Construction of pTnJW4N. *tod*, toluene degradation; *nag*R/P*nag*Aa, regulatory control sequence for naphthalene and phenanthrene degradation from *Comamonas testosteroni* GZ42; oriR, origin of replication derived from plasmid ColE1; IR, inverted repeats from transposon Tn5; Km^R, kanamycin resistance gene; oriT, Tn5 origin of transfer for conjugation; *tnp*, Tn5 transposase gene; d, partly deleted.

Sodium salicylate (1 mM) was added to the minimal medium for induction of the genes involved in 3-methylcatechol formation by *P. putida* MC1 and MC2. *E. coli* and *P. putida* strains were grown at 37 °C and 30 °C, respectively. For 3-methylcatechol production studies, *P. putida* strains were grown in minimal medium to a cell density of 0.20 - 0.60 g/l on the basis of cell dry weight (logarithmic phase), measured as the optical density at 660 nm (OD_{660}). Cells were subsequently diluted to 0.020 g/l with fresh medium containing 1 mM of sodium salicylate and 1.0 - 2.5 mM of toluene in the water phase in airtight Boston bottles (Phase Separations).

Generation and screening of 3-methylcatechol-overproducing pTnJW4N transconjugants

pTnJW4N was introduced into *P. putida* F1 (Zylstra *et al.*, 1988) and F107 (Finette and Gibson, 1988) by triparental mating using pRK2013 as the mobilising plasmid by established procedures (Ditta *et al.*, 1980; Kim and Zylstra, 1995). Kanamycin-resistant colonies were screened for production of 3-methylcatechol using a plate assay (Parke, 1992). This assay is based on the use of a *p*-toluidine reagent that forms a brown precipitate in the agar in the presence of catechols. The colonies were incubated on MM/succ agar in a desiccator in the presence of 1 mM of toluene in the agar medium at equilibrium. The desiccator was kept overnight and colour changes in the agar surrounding the colony were studied. Twenty colonies with the most intense brown halos were tested again and the best producing transconjugants were selected and designated *P. putida* MC1 (derived from *P. putida* F1) and MC2 (derived from *P. putida* F107).

Biotransformations

Biotransformation studies were carried out using growing cells. These experiments were done in airtight Boston bottles (Phase Separations) equipped with Mininert valves (Phase Separations), which allow samples to be taken by a syringe and needle without opening the bottle, to keep the toluene

inside. Boston bottles contained 10 ml of medium and 240 ml of air. Toluene (8 - 20 µl) was pipetted via the wall of the bottle, not directly in the liquid medium, to obtain an initial aqueous toluene concentration between 1.0 and 2.5 mM in the medium. The distribution ratio of toluene over water and air is 3.8 : 1 (w/v) (Amoore and Hautala, 1983) and since there is 24 times more air than water in our batch experiments, the maximum achievable concentration of 3-methylcatechol is 7.5 times the initial aqueous concentration, since toluene is supplied from the gas phase as well (Amoore and Hautala, 1983). Biotransformations were carried out in a horizontally shaking waterbath at 30 °C.

Stability of 3-methylcatechol production was tested by biotransformation of cells that were grown non-selectively for 100 generations. The last culture in this experiment was grown on MM/gluc medium instead of LB, in the presence of inducer of the originally present *tod* genes toluene (0.5 mM) in the 14 hours preceding the biotransformation. This culture was used to inoculate Boston bottles with MM/gluc, toluene, and inducer salicylate for studies on the stability of 3-methylcatechol production.

Analytical methods

OD_{660} measurements for cell densities were carried out in a spectrophotometer (Perkin-Elmer lambda 2 UV/VIS spectrophotometer), using plastic cuvettes (Greiner). Samples for 3-methylcatechol measurement were centrifuged in an Eppendorf centrifuge (Heraeus Instruments, Biofuge Fresco) at 13,000 rpm and 4 °C for 5 minutes. Maximally 200 µl of the supernatant was used in a colorimetric determination of 3-methylcatechol (Arnow, 1937).

CO_2 concentrations were measured via 100 µl headspace analysis in a model 1530A gas chromatograph (Hewlett Packard HP6890 Series GC System) with a Poraplot Q column (Chrompack). An equilibration time of 15 minutes was imposed in 3-methylcatechol toxicity studies, with respect to partitioning of CO_2 between culture and air and with respect to partitioning of 3-methylcatechol in the cell membrane.

RESULTS

Selection for 3-methylcatechol-overproducing pTnJW4N transconjugants

P. putida F1 is able to degrade toluene via *cis*-toluene dihydrodiol and 3-methylcatechol by sequential action of toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase, encoded by *todC1C2BA* and *todD*, respectively (Zylstra *et al.*, 1988). 3-Methylcatechol is further metabolised via the *meta* pathway for catechol degradation. *P. putida* F107 is a 3-methylcatechol-accumulating NTG mutant from F1 that has wild-type todC1C2BAD enzyme levels, but no 3-methylcatechol 2,3-dioxygenase activity (todE) (Finette and Gibson, 1988).

Both strains were used as hosts for introduction of plasmid pTnJW4N, responsible for the formation of 3-methylcatechol from toluene. The genes were cloned downstream of the regulatory control region for naphthalene and phenanthrene degradation from *Comamonas testosteroni* GZ42 (*nag*R/P*nag*Aa) (Figure 1). This promoter is induced by low concentrations of salicylate, a relatively cheap compound that is not utilised by the host strains, leading to high expression levels (Wery and Zylstra, pers. comm.), which is a prerequisite for cost-effective production. The *nag*R/P*nag*Aa-*todC1C2BAD* expression cassette was cloned in between the inverted repeats of the suicide transposon donor TnMod-KmO (Dennis and Zylstra, 1998), resulting in plasmid pTnJW4N. This construct was conjugated into *P. putida* F1 and F107, yielding thousands of transconjugants. Since in pTnJW4N the expression cassette is located on a transposable element, we anticipated stable integration at random sites in the genome. Approximately 2000 transconjugants were screened for 3-methylcatechol formation from toluene on minimal medium agar (Parke, 1992). The best producers, *P. putida* MC1 (from *P. putida* F1) and *P. putida* MC2 (from *P. putida* F107) were genetically analysed.

Genetic analysis of 3-methylcatechol-overproducing transconjugants

Southern analysis was performed on genomic DNA of *P. putida* MC1 and MC2 and their parental strains F1 and F107. The DNAs, uncut or cut by *Not*I, *Pvu*II and *Bam*HI, were hybridised with *todC1* or the kanamycin resistance gene (Km^R) probes after gel electrophoresis and Southern blotting. Uncut pTnJW4N DNA was also analysed. In Figure 2, only the results of the analysis of *P. putida* F107 and MC2 are depicted with *todC1* as the probe. In the lanes containing F107 DNA (Panel (a)) cut with *Not*I and *Pvu*II, hybridising fragments of 3.2 and 4.2 kb, respectively, were visible, which is in agreement with the native tod gene organisation (Lau *et al.*, 1994; Menn *et al.*, 1991; Wang *et al.*, 1995; Zylstra and Gibson, 1989). The *Bam*HI cut DNA gave rise to a 15-kb hybridising DNA fragment and finally a high molecular weight hybridising band was obtained with uncut *P. putida* F107 DNA.

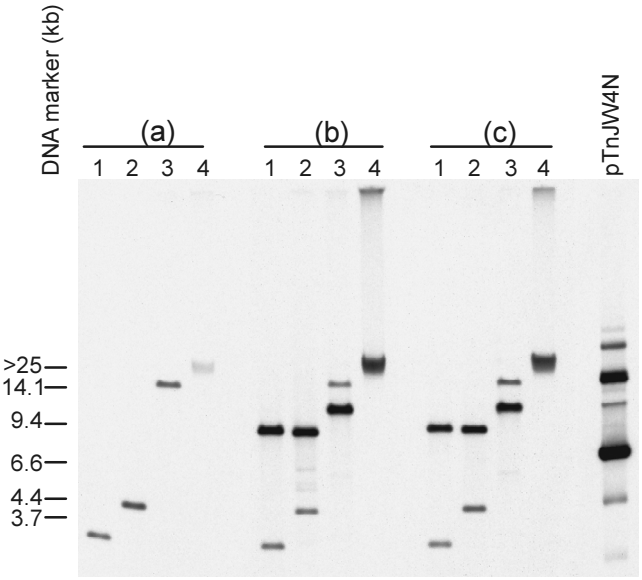

Figure 2 Analysis of total DNA of *P. putida* F107 and MC2 by Southern hybridisation. A 1.2 kb *todC1* DNA fragment was used as the probe. Lanes 1 - 3, DNA digested with *Not*I, *Pvu*II, *Bam*HI, respectively. Lane 4, undigested DNA. (a) Total DNA of *P. putida* F107. (b) Total DNA of *P. putida* MC2. (c) Total DNA of *P. putida* MC2 isolated after 100 generations of non-selective growth. pTnJW4N, undigested pTnJW4N DNA.

Panel (b) (Figure 2) shows a similar analysis with *P. putida* MC2 DNA. As expected, the 3.2-kb *Not*I, the 4.2-kb *Pvu*II and the 15-kb *Bam*HI hybridising native *tod* DNA fragments were visible. In addition to these signals significantly more intense hybridising fragments of 8.7 kb (*Not*I), 8.6 kb (*Pvu*II) and 11 kb (*Bam*HI) were present. These fragments were found to originate from pTnJW4N after reprobing with Km^R (results not shown). In the lane with uncut *P. putida* MC2 DNA only a high molecular weight hybridising band was present with no signs of episomal plasmid DNA, which was clear by comparison with the hybridisation pattern of uncut pTnJW4N. These hybridisation patterns suggest integration of the entire pTnJW4N, rather than a transposition event. Furthermore, the great difference in signal intensity between the native, single copy, hybridising DNA fragments and pTnJW4N indicates that multiple copies are present. It appears that these copies occur in a tandem repeat, since only one plasmid-derived hybridising fragment is obtained with the different digestions. Similar results were obtained with *P. putida* MC1 (results not shown).

We tested the stability of this genotype by long-term non-selective cultivation of *P. putida* MC1 and MC2 in LB medium. After 100 generations of growth, diluted cultures were plated on LB agar and on LB agar plates with kanamycin. Equal numbers of colonies appeared on both plates, indicating that the construct was still present. Additionally genomic DNA was isolated and analysed. Figure 2 (c) shows the results with *P. putida* MC2 DNA. Hybridisation patterns were identical to those obtained from short-term non-selective growth (Panel (b)), indicating that the tandem repeat of pTnJW4N is stably maintained, without loss of copies.

Growth and 3-methylcatechol production in batch cultures

P. putida MC1 and MC2 were compared to NTG mutant F107 with respect to growth and 3-methylcatechol production (Figure 3, Table 1). *P. putida* F1 does not accumulate the product. Biotransformations were carried out with growing cells on minimal medium containing succinate (MM/succ) or glucose (MM/gluc) and salicylate. Toluene was added as the substrate to a concentration in the

aqueous phase of 1.0 mM, which could result in a maximum product concentration of 7.5 mM. Both *P. putida* MC1 and MC2 displayed much higher productivity than mutant *P. putida* F107. Highest product concentrations (Figure 3) and highest specific production rates (Table 1) were obtained with *P. putida* MC2. With this strain up to 7 times higher specific production rates were obtained compared to its parent *P. putida* F107.

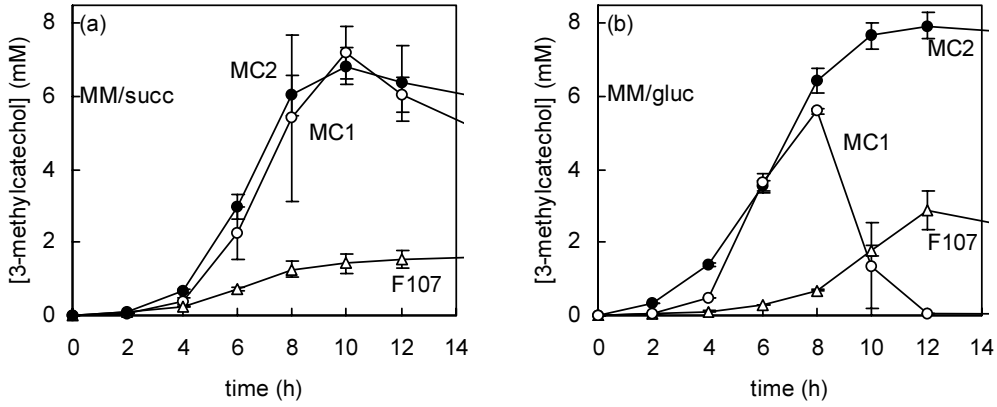

Figure 3 Production of 3-methylcatechol from toluene. 3-Methylcatechol concentration produced by *P. putida* F107 (Δ); MC1 (○); and MC2 (●). Biotransformation media: mineral salts medium with 80 mM of succinate (a) and 80 mM of glucose (b). Data of two independent experiments are presented; error bars indicate ± standard deviation.

We found that growth of the producing strains stopped, independently of growth rate or biomass concentration, shortly after a product concentration of 5 mM was achieved. Surprisingly, the production of 3-methylcatechol by *P. putida* MC1 and MC2 continued for another 2-4 hours, resulting in complete conversion of the substrate.

It has been established that 3-methylcatechol is toxic to microorganisms (Marshall and Woodley, 1995; Robinson *et al.*, 1992), and therefore we studied its effect on growth. We found that the specific growth rate μ decreased by 50 % in medium containing 0.7 mM of 3-methylcatechol. At a concentration higher than 5.5 mM of 3-methylcatechol, no growth was observed (results not shown). Alternatively, headspace CO_2 measurements were carried out in order

to determine metabolic activity of the cells in the presence of 3-methylcatechol (Figure 4). Exponentially growing cells were inoculated in Boston bottles in fresh MM/succ medium containing different concentrations of 3-methylcatechol. CO_2 production decreased rapidly at increasing 3-methylcatechol concentrations and ceased at 14 mM. These results showed that cells were still metabolically active at 3-methylcatechol concentrations that completely inhibited growth, which explains the ability of the cells to still produce the compound at these concentrations.

Table 1 Maximum specific 3-methylcatechol production rates $q_{p,max}$ (µmol min^{-1} g CDW^{-1})‡.

	C source	
	Succinate	Glucose
Strain	$q_{p,max}$	$q_{p,max}$
P. putida F107	13 ± 2	19 ± 9
P. putida MC1	34 ± 0	46 ± 2
P. putida MC2	91 ± 18	105 ± 13

‡ Calculation of q_p (µmol min^{-1} g CDW^{-1}): [3-methylcatechol] increase (µmolar) in two hours, divided by average cell dry weight (g/l) in these two hours, and divided by the time, 120 minutes. Maximum values are depicted. *P. putida* F1 is not included, because it does not accumulate 3-methylcatechol. Initially, 1.0 mM of toluene was present in the culture. Values represent means of two independent duplicate experiments ± standard deviation.

During batch biotransformations, the pH of the MM/succ cultures increased to a final pH of 8.0 - 8.5 and decreased in MM/gluc to pH 6.0, despite buffering. After approximately 8 hours of biotransformation, the succinate medium turned brown as a result of polymerisation of 3-methylcatechol (Finette and Gibson, 1988; Robinson *et al.*, 1992). The product is more stable at low pH. Chemical degradation of 3-methylcatechol is approximately 60 % at pH 8.0, 9 % at pH 7.0, and 3 % at pH 5.8 in 24 hours (results not shown).

From the above, it becomes clear that the rapid decrease of the 3-methylcatechol concentration in the MM/gluc *P. putida* MC1 biotransformation, approximately 76 % in two hours (Figure 3), cannot be explained in terms of chemical degradation only. *P. putida* MC1 is still able to grow on toluene as the sole carbon and energy source (results not shown). Since the induction of

toluene degradation by the native gene products occurs some 7 hours after exposure to toluene (Finette and Gibson, 1988), the sudden drop in 3-methylcatechol levels in the *P. putida* MC1 biotransformations (Figure 3) is most likely a result of enzymatic degradation.

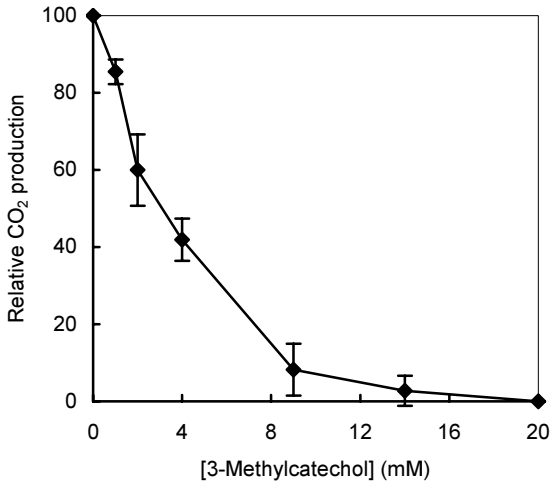

Figure 4 Relative CO_2 production by *P. putida* MC2 as a function of the 3-methylcatechol concentration in MM/succ medium. Headspace CO_2 concentrations after one hour were divided by concentrations after 15 minutes to obtain relative CO_2 production, and were subsequently divided by relative CO_2 production in the blank. Error bars indicate ± standard deviation.

Up to 14 mM of 3-methylcatechol was produced by *P. putida* MC2 in MM/gluc medium in 12 hours when instead of 1.0, 2.5 mM of toluene was added (results not shown).

In conclusion, *P. putida* MC2 is clearly the best strain for bioproduction of 3-methylcatechol. It achieved a specific production rate of 105 µmol min^{-1} g CDW^{-1}.

DISCUSSION

P. putida MC2 has the advantages of a stable genotype and high-rate 3-methylcatechol production. Its specific 3-methylcatechol production rate of 105 µmol min^{-1} g CDW^{-1} is much higher than other catechol-producing systems described so far, ranging from 24 –28 µmol min^{-1} g CDW^{-1} (Robinson *et al.*, 1992; Lilly and Woodley, 1996; Kodama *et al.*, 1996). In addition we found that the catechol-producing properties of *P. putida* MC2 were stable, as it produced 3-methylcatechol at the same rate after prolonged cultivation (over 100 generations) in non-selective medium. However, we unexpectedly found that stable production was not the result of transposition of the 3-methylcatechol expression cassette, but rather was due to the stable maintenance of tandem repeats of the entire construct. We speculate that this is a rare event selected for by the screening of thousands of pTnJW4N transconjugants for the best 3-methylcatechol producer. We found previously that transconjugation of pTnJW4N to *P. putida* S12 did lead to transposition of the transposon part of the construct (Wery *et al.*, 2000). Tandem amplification of a genetic element in *P. putida* F1 transconjugants has recently been described by Ravatn *et al.* (1998).

Plasmid pTnJW4N can be modified for cloning and expression of other genes. Digestion with *Eco*RI and recircularisation results in deletion of the *tod* genes and the 19 bp "outside-end" inverted repeat (Dennis and Zylstra, 1998) creating a unique *Eco*RI cloning site just downstream the *nag*R/P*nag*Aa promoter (Figure 1). The gene of interest, amplified by PCR using primers with the *Eco*RI recognition sequence and a downstream primer that additionally carries the 19 bp "outside-end" inverted repeat could thus be cloned with restoration of the transposon's architecture.

In conclusion, a new approach for enhanced and stable bioproduction of 3-methylcatechol is presented, which is promising for successful application in large-scale production systems.

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Chapter 3

Optimisation of microbial 3-methylcatechol production as affected by culture conditions

ABSTRACT

Pseudomonas putida MC2 can produce catechols. The accumulation and the specific and volumetric production rates of 3-methylcatechol from toluene were studied. Production rates were shown to depend on growth medium, pH and toluene concentration. Minimal glucose medium and rich LB medium were the best growth substrates for 3-methylcatechol production. A lower pH often resulted in slower growth and a higher specific production rate, but a lower volumetric production rate. Specific production rates also increased at higher initial toluene concentrations. In conclusion, the best process conditions in terms of substrate conversion and specific production rate are found in glucose medium at an initial aqueous toluene concentration of 1.0 mM and an initial pH of 6. At pH 7 and 2.0 mM of toluene, more product was accumulated at a lower specific rate, but at a higher volumetric production rate.

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INTRODUCTION

For pharmaceutical synthesis, 3-substituted catechols are valuable precursors (Van Scharrenburg and Frankena, 1996; Ennis and Ghazal, 1992). Chemical production of 3-substituted catechols is complex and is hampered by a low overall yield (Van Scharrenburg and Frankena, 1996). Microbial production may be an attractive alternative, since certain microorganisms specifically accumulate catechols (Robinson *et al.*, 1992; Marshall and Woodley, 1995; Held *et al.*, 1998, Rodriguez *et al.*, 1999).

Bioproduction of 3-methylcatechol from toluene has been studied as a model conversion for the production of 3-substituted catechols in general (Hüsken *et al.*, 2001). Recently, we described the construction of a recombinant *Pseudomonas putida* strain, *P. putida* MC2, that accumulates high concentrations of 3-methylcatechol from toluene (Figure 1) at high rates (Hüsken *et al.*, 2001).

In this paper, we investigated the 3-methylcatechol production profile of this strain as affected by culture conditions. Maximum specific production rates were found to be highly dependent on the growth substrate, pH and toluene concentration. Accumulation of 3-methylcatechol and volumetric production rates were also studied. Criteria for optimisation of 3-methylcatechol production are discussed.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Pseudomonas putida MC2 was previously constructed and characterised (Hüsken *et al.*, 2001). This strain is derived from *P. putida* F107 (Finette and Gibson, 1988), a mutant containing the *tod* toluene degradation pathway, but lacking the enzymes for degradation of 3-methylcatechol; it thus accumulates this product. *P. putida* MC2 was obtained by introduction of an extra set of the *todC1C2BAD* genes for 3-methylcatechol production under control of a strong

heterologous salicylate-induced promoter in *P. putida* F107. *Pseudomonas putida* MC2 was shown to be a stable 3-methylcatechol producer, without the need for antibiotics in the medium, and was unable to enzymatically degrade the product (Hüsken *et al.*, 2001).

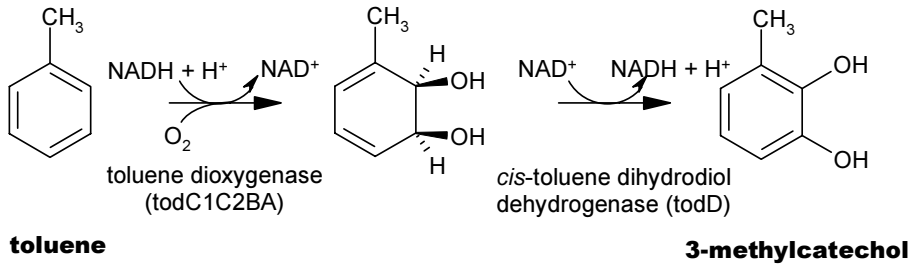

Figure 1 Enzymatic conversion of toluene to 3-methylcatechol in *Pseudomonas putida* F1 (Zylstra *et al.* 1988) and in *P. putida* MC2.

For growth, LB broth (Sambrook *et al.*, 1989) was utilised as rich medium. For incubations, LB medium or mineral salts medium (Hartmans *et al.*, 1989) with a 60 mM phosphate buffer at pH 7 was used. HCl or NaOH solution (6.0 M) were used to adjust the pH when necessary. As carbon and energy source, 80 mM of sodium succinate or glucose was added to mineral medium. Liquid precultures contained 0.5 mM of toluene for induction of the native *tod* genes, because this induction takes about 7 hours (Finette and Gibson, 1988), but did not contain salicylate, which has a shorter response time of 1 – 2 hours.

Small-scale fermentations

The liquid precultures were used to inoculate fresh medium in airtight 250-ml Boston bottles (Phase Separations), containing 240 ml of gas and 10 ml of culture at 0.017 g cell dry weight (CDW) / l (± 0.005). The inducer salicylate was added to a concentration of 1.0 mM for induction of the cloned 3-methylcatechol-production genes. The reaction substrate toluene (8 - 20 µl)

was added, resulting in an initial aqueous toluene concentration between 1.0 and 2.5 mM in the liquid medium. The distribution ratio of toluene over water and air is 3.8 : 1 (w/v) (Amoore and Hautala, 1983) and since there is 24 times more air than water in our batch experiments, the maximum achievable concentration of 3-methylcatechol is 7.5 times the initial aqueous concentration, because toluene is supplied from the gas phase as well (Amoore and Hautala, 1983). Small-scale fermentation studies were done in a horizontally shaking waterbath at 30 °C.

Methods

For measurements of cell density and product concentration, a Perkin-Elmer lambda 2 UV/VIS spectrophotometer and 1-cm cuvettes were used. Cell densities were measured at 660 nm, while for 3-methylcatechol concentrations Arnow's method at 510 nm was used (Arnow, 1937).

RESULTS

Production of 3-Methylcatechol: Effect of growth substrate

Pseudomonas putida MC2 is a stable recombinant strain (Hüsken *et al.*, 2001). If the inducer salicylate is present in the medium, 3-methylcatechol is accumulated rapidly. Small-scale fermentations of *P. putida* MC2 with toluene and salicylate in different growth media were employed to study the effect on 3-methylcatechol production. Results are summarised in Table 1.

It was found that growth substrates influenced specific 3-methylcatechol production rates considerably. Glucose was the best growth substrate for this production process (Table 1). Specific production rates were high (up to 235 µmol min^{-1} g CDW^{-1}) and at pH 6, 7 and 8 and an initial aqueous toluene concentration of 1.0 mM, all toluene was converted to 3-methylcatechol (Table 1).

In all media tested, 3-methylcatechol production proceeded for 2 – 3 hours after cells entered stationary phase, and finally stopped.

Effect of pH

Succinate and LB media became alkaline (pH rose to 8 – 8.5) during growth of *P. putida* MC2, while glucose medium became acidic (pH 5 – 6), despite buffering. In mineral media with succinate or glucose, growth was slower at an initial pH of 6 than at pH 7. Nevertheless, 3-methylcatechol production curves were similar, indicating that cells were more actively producing at pH 6. This difference in specific activity was not caused by more efficient enzyme induction due to the presence of more uncharged salicylate at lower pH (results not shown).

In LB medium at an initial pH of 5, the pH increased rapidly to 7; growth and production rates were comparable to pH 7. At an initial pH of 8, growth and production were also comparable while the pH did not rise much. However, at pH 8 or higher the product is rather unstable (Hüsken *et al.*, 2001).

In contrast to LB and succinate medium, the pH decreased during growth of *P. putida* MC2 in glucose medium. At pH 5, growth was negligible and 3-methylcatechol was produced at a low volumetric production rate of 0.06 g l^{-1} h^{-1} and a specific production rate of 138 µmol min^{-1} g CDW^{-1} (Table 1). At pH 6, the cells produced 3-methylcatechol much faster at a specific production rate of 235 µmol min^{-1} g CDW^{-1}, which is the highest value we measured in these experiments.

Our operational window for high 3-methylcatechol productivity is therefore limited to an initial pH between 5 and 7 on LB medium and between 6 and 8 on glucose medium (Table 1).

Table 1 Production of 3-methylcatechol (3-MC), conversion, biomass concentration and maximum specific and volumetric production rates by *P. putida* MC2 as a function of medium, initial pH and initial aqueous toluene concentration. Initial cell density 0.017 g l^{-1}.

growth medium	initial pH	initial [toluene] (mM)[a]	$[3\text{-MC}]_{max}$ (mM)[a, b]	CDW (g l^{-1}) (12 h)	$q_{p,\,max}$ (µmol min^{-1} g CDW^{-1})[c]	$Q_{p,\,max}$ (g l^{-1} h^{-1})
succinate	6.0	1.0	4.5 (60)	0.16	162	0.10
	7.0	1.0	7.1 (94)	0.39	91[d]	0.19
		2.5	6.6 (88)	0.19	153	0.15
glucose	5.0	1.0	4.2 (55)	0.06	138	0.06
	6.0	1.0	7.2 (96)	0.14	235	0.17
		2.0	3.5 (23)	0.04	243	0.07
	7.0	1.0	8.0 (106)	0.38	105[d]	0.18
		2.0	14.2 (95)	0.32	150	0.31
		2.5	13.6 (72)	0.26	181	0.31
	8.0	1.0	7.5 (100)	0.77	72	0.24
LB	5.0	1.0	7.4 (98)	0.67	34	0.16
	6.0	2.5	7.5 (40)	0.44	41	0.15
	7.0	1.0	7.7 (102)	1.36	24	0.18
		2.5	10.2 (54)	0.88	50	0.34
	8.0	1.0	6.4 (85)	1.09	22	0.15

Values represent means of two independent duplicate experiments.
[a] Aqueous concentration. Since toluene is mainly present in the gas phase, a higher product concentration can be obtained in the culture medium than the initial toluene concentration.
[b] Toluene conversion (%) is shown in brackets.
[c] Calculation of q_p (µmol min^{-1} g CDW^{-1}): [3-methylcatechol] increase (µmolar), divided by time (minutes), and divided by cell dry weight (g l^{-1}). This calculation equals $q_p = \mu \cdot Y_{p/x}$. Maximum q_p values are depicted.
[d] Data from Hüsken *et al.* (2001).

Effect of initial toluene concentration

Toluene is needed as substrate for the reaction, but toluene is a toxic compound for microorganisms and inhibits growth of the cells (Sikkema *et al.*, 1995). Our efforts to find optimal production were focused on the right balance between substrate availability and substrate toxicity. Initial experiments were done at a starting concentration of toluene of 1.0 mM in the water phase as growth was hardly affected under these conditions. At an initial toluene concentration of 2.5 mM, the specific growth rate decreased by 50%.

More product (up to 14.2 mM) was formed at higher toluene concentrations on glucose and LB medium at pH 7 (Table 1). Maximum specific production rates almost doubled if the toluene concentration was increased from 1.0 to 2.5 mM (Table 1). At an initial concentration of toluene of 1.0 mM, toluene depletion limited total 3-methylcatechol accumulation.

Increasing the initial toluene concentration from 2.0 to 2.5 mM did not result in a higher accumulation of 3-methylcatechol (Figure 2). At 1.0 and 2.0 mM, more than 95% of toluene was converted to 3-methylcatechol, while the conversion at 2.5 mM of toluene was 72% in glucose medium (Amoore and Hautala, 1983). At 2.5 mM, the maximum specific production rate was higher (181 μmol min^{-1} g CDW^{-1}) than at 2.0 mM (150 μmol min^{-1} g CDW^{-1}). However, accumulation of the toxic product stopped production (Figure 2), and the remaining toluene was not converted.

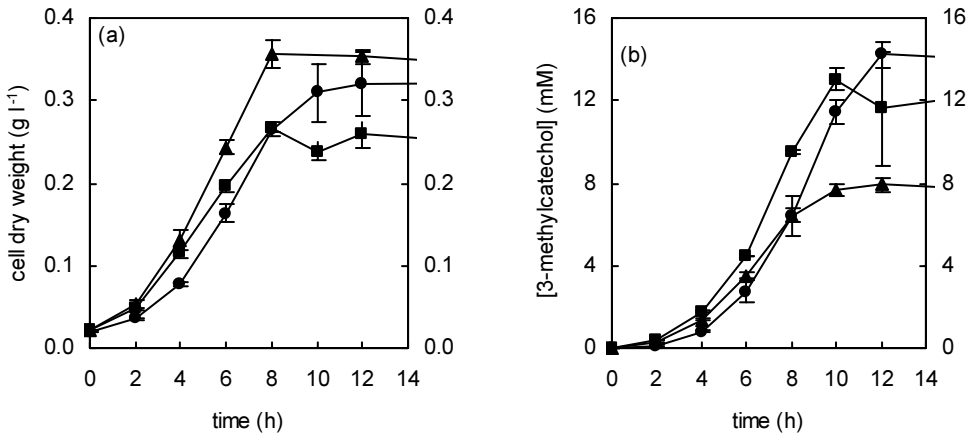

Figure 2 Growth (a) and accumulation of 3-methylcatechol (b) by *P. putida* MC2 on glucose medium at an initial pH of 7, with varying initial aqueous toluene concentrations. 1.0 mM (▲), 2.0 mM (●), and 2.5 mM of toluene (■).

DISCUSSION

To optimise any production process, clear criteria are ultimately required. It is imperative to establish the economic relevance of production rates and product concentrations and thus to choose the appropriate optimisation parameter. In the present case, growth media, the inducer salicylate and reaction substrate toluene are inexpensive. The question is whether the overall production costs are determined by the specific production rate (biocatalyst costs), by the volumetric production rate (reactor costs), or by the product concentration (downstream costs).

For a high specific 3-methylcatechol production rate (μmol min^{-1} g CDW^{-1}), the optimal conditions were glucose medium and pH 6 (Table 1). The initial toluene concentration was not rate-determining here (235 μmol min^{-1} g CDW^{-1} at 1.0 mM and 243 μmol min^{-1} g CDW^{-1} at 2.0 mM). Under these conditions, growth was modest, but the production by the active cells was high, resulting in high specific production rates.

Highest volumetric production rates (g l^{-1} h^{-1}) were obtained in succinate medium at pH 7, in glucose medium at pH 6 and 7, and in LB medium at pH 7 (0.17 – 0.19 g l^{-1} h^{-1} at 1.0 mM of toluene; Table 1). At a toluene concentration of 2.5 mM, a higher rate was obtained on glucose and LB media (0.31 – 0.34 g l^{-1} h^{-1}; Table 1). Glycerol medium at pH 6 was also tested but gave a very low volumetric production rate (0.04 g l^{-1} h^{-1}). In LB medium, cells grew fast with low specific 3-methylcatechol production rates. However, the volumetric production rate was high and similar to that in succinate and glucose media (Table 1).

Volumetric production rates were improved by using higher initial biomass concentrations. On LB medium at an initial biomass concentration of 1.6 g l^{-1} (final CDW 2.1 g l^{-1}) and with 2.5 mM of toluene, specific production rates were somewhat lower (40 µmol min^{-1} g CDW^{-1}), but the volumetric 3-methylcatechol production rate was improved to 0.60 g l^{-1} h^{-1} as compared to incubations with lower biomass concentrations.

If a high 3-methylcatechol concentration is essential, glucose should be used as growth substrate, at an initial pH of 7 and an initial toluene concentration of 2.0 mM. It is clear from Figure 2 that the accumulation of the toxic product affected bacterial growth. Maximum conversion was achieved after 12 hours (Figure 2).

To obtain even higher product concentrations, a continuous toluene feed might improve our results by minimising substrate inhibition. The use of an adsorbent or a second organic phase as substrate provider and product extracter may obviate inhibitory product concentrations in the culture medium. Toxicity is a serious problem as illustrated by worse growth and a decreased conversion at higher toluene concentrations, although the q_p was higher when more toluene was present at pH 7 (Table 1).

Adsorption to remove toxic products and their inhibitory effects during incubation was shown to be beneficial for the yield (Robinson *et al.*, 1992; Held *et al.*, 1998; Held *et al.*, 1999). A higher 3-methylcatechol concentration and toluene conversion were obtained when granular activated charcoal was used for continuous product removal (Robinson *et al.*, 1992). For the production of

other 3-substituted catechols, a fluidised bed for *in situ* product recovery was developed (Held *et al.*, 1998; Held *et al.*, 1999). The adsorbent Amberlite™ XAD-4 was used and gram amounts of different 3-substituted catechols were produced.

The use of a second, organic phase to provide the substrate and to extract the product was shown to improve product accumulation (Wery *et al.*, 2000). Toxicity problems of both substrate and product may be resolved in this approach (De Bont, 1998), but it is important to find a solvent that in itself is not too toxic for the cells. *P. putida* MC2 is tolerant to some organic solvents that might be of use in this context, where an appropriate balance between solvent toxicity and product partitioning is the main issue.

Future experiments will therefore focus on organic/aqueous two-phase systems for integrated production and downstream processing with low substrate and product concentrations in the culture medium.

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Zylstra GJ, Gibson DT (1989) Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J Biol Chem 264: 14940-14946

Chapter 4

Integrated bioproduction and extraction of 3-methylcatechol

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.

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INTRODUCTION

In biocatalysis, many processes are limited by inhibition of substrates and/or products (De Bont, 1998; Collins *et al.*, 1995; Marshall and Woodley, 1995; Robinson *et al.*, 1992). 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; Hartog and Wouters, 1988; Van Scharrenburg and Frankena, 1996), but they are difficult to synthesize chemically (Held *et al.*, 1999; Van Scharrenburg and Frankena, 1996). We studied the model conversion of toluene to 3-methylcatechol by *Pseudomonas putida* MC2 in aqueous media (Hüsken *et al.*, 2001, 2002). 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 *Pseudomonas 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 *Pseudomonas 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^{TM} 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 (Collins *et al.*, 1995; Collins and Daugulis, 1999a, 1999b; De Bont, 1998; Brink and Tramper, 1985; Harbron *et al.*, 1986; Tramper *et al.*, 1992; Wery *et al.*, 2000). Exchange kinetics and optimisation 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; Isken *et al.*, 1999; Vermuë *et al.*, 1993).

Toxicity of organic solvents for micro-organisms depends on the hydrophobicity of the solvent (Weber and De 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 (De Bont, 1998).

The log$P_{o/w}$ of 1-octanol is 2.92 (Osborne *et al.*, 1990), indicating its toxicity (De 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 favour 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 *Pseudomonas 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) the overall 3-methyl-catechol 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-liter 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.

MATERIALS AND METHODS

Pseudomonas putida MC2

Pseudomonas putida MC2 was previously constructed and characterised (Hüsken *et al.*, 2001). 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.

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}).

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).

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 minimise oxygen limitation.

Fermentations

The bioreactor used was an Applikon 1.8-litre flat-bottom fermentor (Applikon Dependable Instruments, Schiedam, The Netherlands) and contained 0.80 litre 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 one minute 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 sample pipe. After that, a sample was extracted from the reactor.

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 colour 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 13,000 rpm and 4 °C for 3 minutes. 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 minutes 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 x 4.6 mm (L x ID; Chrompack Chromsep, Zorbax 7), applying a flow of 1.2 ml min^{-1} of eluents, containing 0.1 % trifluoroacetic acid in a 1 : 1 methanol – water matrix. 3-Methylcatechol was detected in a UV spectrophotometer at 210 nm (retention time 5 minutes). These measurements were carried out as a check for partitioning.

RESULTS AND DISCUSSION

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.*, 2001); it is thus a good candidate for two-phase 3-methylcatechol production.

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 (Figure 1).

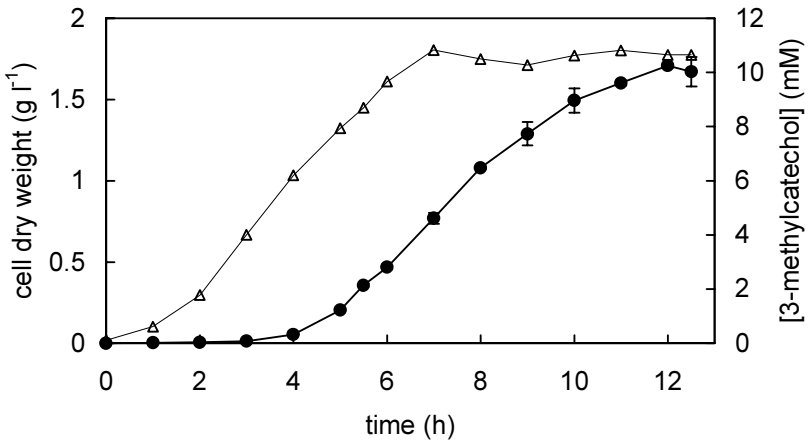

Figure 1 Typical graph of growth of *Pseudomonas putida* MC2 (Δ) and accumulation of 3-methylcatechol (●) in a bioreactor with aqueous medium and a constant toluene concentration of 1.0 mM. Error bars indicate standard deviation between two samples.

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), 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.*, 2002). 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.*, 2001), while above 6 mM, no growth was observed at all, although cells were still active. This agrees with the results of Figure 1.

Elevated product concentrations thus inhibit growth and consequently production in single-phase 3-methylcatechol production processes.

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.

Small-scale two-phase optimisation 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 analysed 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.

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 (Figure 2); this value was chosen for the subsequent bioreactor experiments.

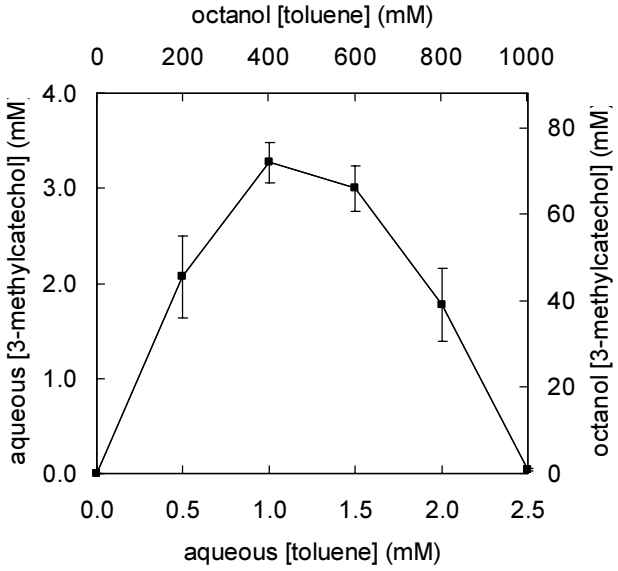

Figure 2 Accumulated aqueous and octanol concentrations of 3-methylcatechol as a function of aqueous and octanol toluene concentration in a 1 : 1 octanol/aqueous two-phase system (small scale).

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.*, 2002), but in two-phase Boston bottles this medium showed a long lag time of 40 hours (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: fourfold 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 colour at the end of the incubation due to e.g. polymerisation. This implies that more product was accumulated than was measured.

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

Phase ratio (octanol:LB, v/v)	10 ml scale, shake flasks		0.80 litre 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	ND

[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-litre 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).
ND: not determined.

Organic/aqueous two-phase fermentations

Small-scale experiments were scaled up to 0.80-litre experiments in a bioreactor, with the advantages of pH control, constant toluene concentrations and easier sampling. The bioreactor contained 0.80 litre 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.

Figure 3 shows experiments in which 3-methylcatechol concentrations *vs.* time were measured in a 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 hours (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.

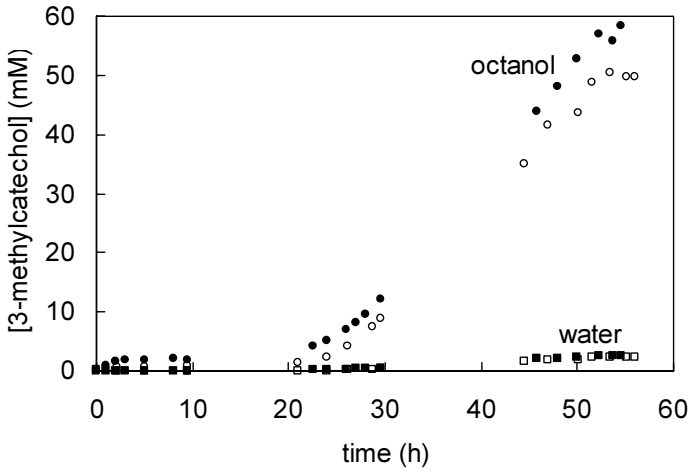

Figure 3 Accumulation of 3-methylcatechol in organic/aqueous two-phase system. In the bioreactor, 40 vol % of octanol was present. Results of two independent experiments are depicted. ●, ○: 3-methylcatechol concentration in octanol phase; ■, □: concentration in aqueous phase.

The time to attain the final product concentration (53 hours, Figure 3) was longer than in single-phase systems (12 hours, Figure 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-liter 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-liter 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 grey, and via brownish red it changed to dark brown at the end of the incubation, probably as a result of product polymerisation (Robinson *et al.*, 1992; Shirai, 1987). In a buffer at pH 7, 9 % of 3-methylcatechol disappears in 24 hours (Hüsken *et al.*, 2001). 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.

Optimisation 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, Figure 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.

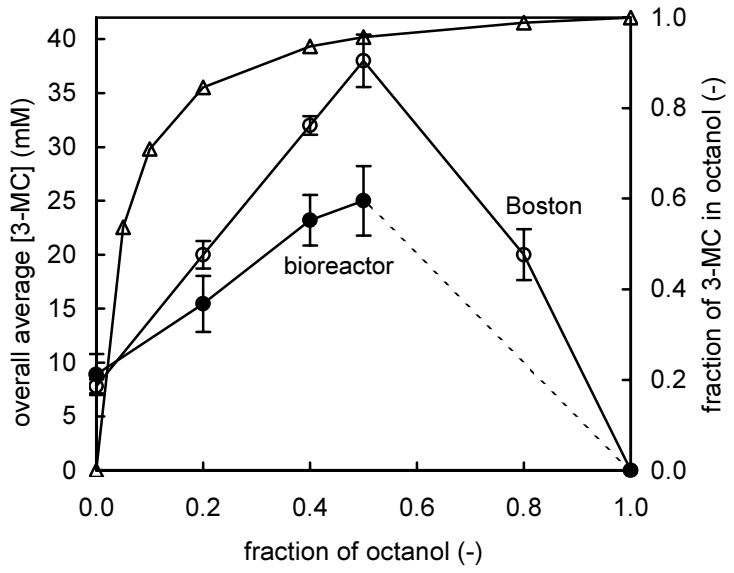

Figure 4 Average product concentration over total volume as a function of phase ratio octanol/water, both in small-scale Boston bottles (○) and in bioreactor experiments (●). Error bars indicate standard deviations. (△) Fraction of product in organic phase as a function of phase ratio octanol/water.

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 (Figure 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 Figure 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 disodium 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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Chapter 5

Membrane-facilitated bioproduction of 3-methylcatechol in an octanol/water two-phase system

ABSTRACT

Bioproduction of 3-methylcatechol from toluene by *Pseudomonas putida* MC2 was studied in the presence of an additional 1-octanol phase. This solvent was used to supply the substrate and extract the product, in order to keep the aqueous concentrations low. A hollow-fibre membrane kept the octanol and aqueous phase separated to prevent phase toxicity towards the bacterium. Volumetric production rates increased approximately 40% as compared to two-phase 3-methylcatechol production with direct phase contact. Preliminary investigations on downstream processing of 3-methylcatechol showed that 1 M of sodium hydroxide selectively extracted the disodium salt of 3-methylcatechol into an aqueous phase.

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INTRODUCTION

Catechol and its derivatives are important chemicals that are used in the manufacture of synthetic flavours such as vanillin (Shirai, 1986) and are used as precursors for pharmaceutical production (Ennis and Ghazal, 1992; Van Scharrenburg and Frankena, 1996). They are difficult to synthesize chemically (Held *et al.*, 1999; Van Scharrenburg and Frankena, 1996).

Some microorganisms are known to produce catechols (Held *et al.*, 1999; Robinson *et al.*, 1992; Haigler and Spain, 1991). As a reference system, we have studied the conversion of toluene to 3-methylcatechol by *Pseudomonas putida* MC2 in aqueous media (Hüsken *et al.*, 2001a, 2002). The product is not converted or consumed by these cells. For the production of 3-methylcatechol, growing cells are needed, but in single-phase media growth stopped early due to the rising product concentration, therewith limiting production levels. This is a very general phenomenon. Many bioprocesses are limited by inhibition of the biocatalyst by substrate and/or product (De Bont, 1998; Collins *et al.*, 1995; Robinson *et al.*, 1992).

To overcome inhibition in a biocatalytic production process and to enhance final product concentrations, an organic solvent can be utilised. The organic solvent serves as a reservoir for the substrate and, ideally, extracts the product (De Bont, 1998; Schmid *et al.*, 2001). Therefore, we previously have studied 3-methylcatechol production by *Pseudomonas putida* MC2 in an organic/aqueous two-phase system, using 1-octanol as an organic phase for the supply of the substrate and the extraction of the product (Hüsken *et al.*, 2001b). Compared to aqueous processes, production was enhanced, although octanol was in direct contact with the aqueous culture medium (Hüsken *et al.*, 2001b). Overall (over total liquid volume) 3-methylcatechol concentrations of 25 mM were obtained in a 1 : 1 two-phase system (48 mM in octanol and 2.2 mM in aqueous culture medium), while in aqueous reactions 10 mM of 3-methylcatechol was produced.

P. putida MC2 is known to be tolerant to octanol and it is active in the presence of this solvent (Hüsken *et al.*, 2001b), as was also found for *P. putida* DOT-T1E (Ramos *et al.*, 1998). However, an extended lag time in

organic/aqueous two-phase systems was observed. In spite of the fact that the bacterium is tolerant to octanol, the organic/aqueous interface can have a detrimental effect on the cells (Isken and De Bont, 1996). This effect is known as phase toxicity (Bar, 1988; Weber and De Bont, 1996). We anticipated that growth of the cells and production of 3-methylcatechol in the presence of octanol could be further improved if phase toxicity could be overcome. Moreover, it is not easy to efficiently remove the product from the organic/aqueous two-phase bioreactor as described by Hüsken *et al.* (2001b). For these reasons, in the present investigation a membrane was placed between the aqueous and the organic phase to prevent direct contact of the cells with the organic phase. An additional advantage is that the two phases remain separated and this facilitates downstream processing considerably.

Choi and co-workers (1999, 2000) showed that the use of a hydrophilic hollow-fibre membrane bioreactor was advantageous in the microbial resolution of 1,2-epoxyhexane. Use of the membrane prevented direct contact between yeast cells and solvent phase, therewith minimising inhibitory solvent effects on biocatalysis (Choi *et al.*, 1999). Furthermore, product inhibition was overcome. The same advantages were found by Molinari *et al.* (1997) in an extractive conversion process for the production of isovaleraldehyde. A hydrophobic membrane kept an organic solvent phase separated from an aqueous phase containing the bacteria.

In this paper, we describe the use of a hydrophobic hollow-fibre membrane in the bioconversion of toluene to 3-methylcatechol by designer biocatalyst *P. putida* MC2. Cells are present in an aqueous culture medium in a bioreactor. This culture is pumped through the fibres of the membrane module. The octanol phase that extracts the product is co-currently pumped through the shell side. The membrane keeps both phases separated. In this experimental set up, cells will hardly come into direct contact with octanol and will thus barely be subjected to phase toxicity. We investigated whether this resulted in higher volumetric productivities. In addition, purification of the product from octanol was studied and a conceptual process design for the continuous production of 3-methylcatechol is discussed.

MATERIALS AND METHODS

Culture conditions

Pseudomonas putida MC2 was previously constructed and characterised (Hüsken *et al.*, 2001a). LB medium (Sambrook *et al.*, 1989) was used in agar plates, containing 50 µg kanamycin per ml, to grow the bacterium overnight. A colony from this plate was used to inoculate LB precultures, containing 0.5 mM of toluene, but lacking both the antibiotic and the inducer salicylate. Toluene was present to adapt the cells to the substrate, while salicylate was only added during the production process.

Precultures were grown to 1.1 g cell dry weight (CDW) per liter. They were used (20 ml) to inocculate the bioreactor, an Applikon 1.8 liter flat-bottom bioreactor (Applikon Dependable Instruments, Schiedam, The Netherlands), in which 1.2 liter of LB medium was stirred (400 – 700 rpm). This aqueous medium contained 1.0 mM of salicylate, to induce the enzymes that are responsible for toluene degradation to 3-methylcatechol (Hüsken *et al.*, 2001a), and 0.1% of antifoam agent polypropylene glycol (average MW = 2000). The bioreactor was pH-controlled at pH 7.0, using 1.0 M HCl. A feed of 15-fold concentrated LB medium (containing NaCl at normal concentration) was added at a rate of 2 ml h^{-1}. The medium was aerated with toluene-containing air, corresponding to an equilibrium concentration of toluene in the medium of 1.0 mM (Hüsken *et al.*, 2001b). The air was bubbled through a sparger at 400 ml min^{-1}, and the off-gas was led through a condensor. All experiments were carried out at 30 °C.

Membrane module and experimental set-up

A hollow-fibre polypropylene membrane module (Microdyn, MD 020 CP 2N, Wuppertal, Germany) containing 40 capillaries (internal diameter of 1.8 mm) was used. The effective surface area was 0.1 m^2 and the average pore size 0.2 µm.

Before use, the membrane was modified as described by Schroën *et al.* (1993). Block co-polymer F108 (ICI, Rotterdam, The Netherlands) was anchored on the membrane surface, at the fibre side. This polymer consisted of a hydrophobic anchor group of polypropylene oxide and two hydrophilic polyethylene oxide buoy groups that prevented membrane fouling (Schroën *et al.*, 1993).

Bioconversion was allowed to take place for two hours without circulation of either of the liquid phases. Aqueous medium from the bioreactor (1200 ml), including bacteria, was pumped through the membrane fibres at 250 ml min^{-1}. Octanol (600 ml) containing 400 mM of toluene, was simultaneously pumped to the shell side of the membrane and returned to the storage vessel at a flow rate of 125 ml min^{-1}. After each experiment, the Viton tubes used to pump both phases to the membrane module were replaced, to prevent deterioration of the tubes inside the pumps and consequently leakage of solvent or aqueous phase. In between experiments, the fibres of the module were rinsed with water. The membrane module was used for ten experiments before disposal.

The culture medium was pumped through the fibres, and not through the shell side of the membrane, to limit the residence time distribution of the bacteria in the module. The aqueous and the organic phase were pumped in co-current mode to prevent a pressure drop across the membrane, and therewith related, permeation of culture or organic phase.

Downstream processing of 3-methylcatechol

Octanol, containing 400 mM of toluene and (initially) 60 mM of 3-methylcatechol, was extracted by vigorous shaking with 1.0 M of sodium hydroxide at 30 °C. Different phase ratios and contact times were applied. Samples were centrifuged at 30 °C for 5 seconds at 13,000 rpm in an Eppendorf centrifuge (Heraeus Instruments, Biofuge Fresco), to separate both phases quickly. Subsequently, 3-methylcatechol was measured in the alkaline aqueous phase, as described in the analytical methods.

Analytical methods

Cell densities in aqueous systems were measured at 20 °C in a spectrophotometer (Perkin-Elmer lambda 2 UV/VIS spectrophotometer), at 660 nm (OD_{660}) in 1.0-cm plastic cuvettes (Greiner). It was checked if the product interfered with this measurement and this was not the case.

Prior to measurement of their 3-methylcatechol concentration, aqueous samples were centrifuged in an Eppendorf centrifuge (Heraeus Instruments, Biofuge Fresco) at 13,000 rpm and 4 °C for 3 minutes. Up to a maximum of 200 µl of the supernatant was used in a colorimetric test. The concentration was measured at 510 nm (Arnow, 1937), using a calibration curve (extinction coefficient of 3-methylcatechol in LB medium 1.65 mM^{-1} cm^{-1}).

Product concentrations in octanol were also determined, to check partitioning between both phases. The partition coefficient of 3-methylcatechol over 1-octanol and LB medium was found to be 22 m^3 LB / m^3 octanol (Hüsken *et al.*, 2001b). By vortexing an octanol sample with an equal volume of fresh LB-medium and measuring the equilibrium product concentration in the LB medium (as described above), the 3-methylcatechol concentration in the octanol sample was calculated.

RESULTS AND DISCUSSION

Growth and bioproduction of 3-methylcatechol

In the present study, biphasic biocatalysis without direct phase contact was chosen. A culture of growing *P. putida* MC2 in LB medium in a bioreactor was producing 3-methylcatechol from toluene. Simultaneously, octanol was used to extract the toxic product from the aqueous phase, and a hydrophobic membrane was used to keep both phases separated.

Although phase toxicity for the cells was strongly reduced, molecular toxicity (Bar, 1988; Vermuë *et al.*, 1993; Weber and De Bont, 1996) would be possible, since octanol molecules accumulated in the aqueous phase until

saturation. A phase ratio of 1 (organic) : 2 (aqueous) (v/v) was applied. Biomass and 3-methylcatechol concentrations were monitored in the aqueous phase. The specific growth rate in the membrane bioreactor was comparable to growth in a single-phase (aqueous) process (Hüsken *et al.*, 2001b). Also in growth experiments where a sub-saturated concentration of octanol was added, no change was found in the specific growth rate of the cells (results not shown). Therefore, it could be concluded that growth was not inhibited by molecular octanol toxicity. In the membrane production process, growth and production started almost immediately, *i.e.* at t = 0.5 h (Figure 1). This is in sharp contrast to our previous findings for a two-phase process with direct phase contact (Hüsken *et al.*, 2001b). In the latter case, a long lag time of up to 24 hours occurred during which no product accumulated.

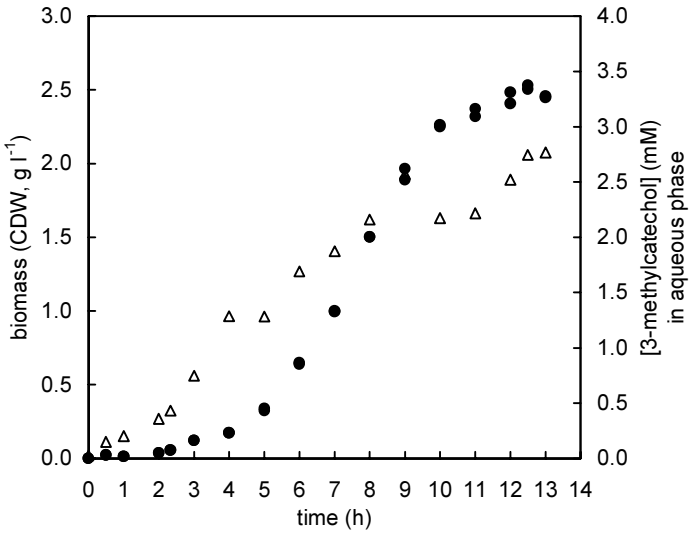

Figure 1 Cell density and 3-methylcatechol concentration in aqueous phase. A membrane kept the octanol phase (600 ml) separate from the culture medium (1200 ml). Δ cell density (g CDW l^{-1}), ● 3-methylcatechol concentration in LB medium (mM).

In the membrane process, growth was exponential for 4 hours (0.2 mM of product), after which the growth rate decreased. After 12 hours, the biomass

concentration was 2.1 g CDW l^{-1} and 3.3 mM 3-methylcatechol was present in the aqueous medium (Figure 1). In the two-phase process with direct phase contact, the highest aqueous product concentration was 3.0 mM after approximately 24 hours, at a phase ratio of 1 (organic) : 4 (aqueous) (v/v). This aqueous concentration is comparable to that found in the present membrane system albeit that in the membrane system, the overall volumetric production rate was higher.

In the octanol phase of the membrane process, 17 mM of product was found through partition data, as described in the Materials and Methods section. Equilibrium of 3-methylcatechol over both phases was not reached within the process time of 12 hours, because the ratio between the concentrations in the organic and the water phase was only 5 (apparent partitioning) where it should have been 22 for equilibrium (Hüsken *et al.*, 2001b). The membrane resistance was higher in the presence of biomass than in experiments without cells. Concentration polarisation of bacteria (Mulder, 1996) at the membrane surface, and/or fouling of the membrane despite its treatment with block co-polymer F108, might be causes of the decreased extraction. Also, accumulation of antifoam agent PPG-2000 at the hydrophobic membrane surface may have occurred and have caused a higher mass transfer resistance. In the absence of membrane fouling, if any, the product would be extracted faster, resulting in a slower rise of the aqueous (toxic) product concentration, which could imply a higher productivity. Production of 3-methylcatechol in our process was apparently faster than extraction to octanol through the membrane.

A larger membrane surface area would provide a means to properly adjust the extraction rate to the production rate. Nevertheless, the membrane process was advantageous with respect to volumetric production rates. Production was faster than in the process with direct phase contact (Hüsken *et al.*, 2001b). In the latter process, at the optimal 1 : 1 phase ratio, 48 mM accumulated in the octanol phase in 53 hours, corresponding to 0.91 mol h^{-1} m^{-3} octanol (Figure 2). In aqueous reactions, a somewhat lower rate of 0.79 mol h^{-1} m^{-3} LB was found. In the membrane process, however, 17 mol m^{-3} accumulated in octanol in 12 hours, yielding 1.4 mol h^{-1} m^{-3} octanol (Figure 2). In spite of the fact that

the overall volumetric production rate decreased somewhat compared to the aqueous system (Figure 2), the process was faster than without a membrane, and additionally, downstream processing of the octanol phase was facilitated. The advantages for downstream processing of the membrane process are reported in the next paragraph.

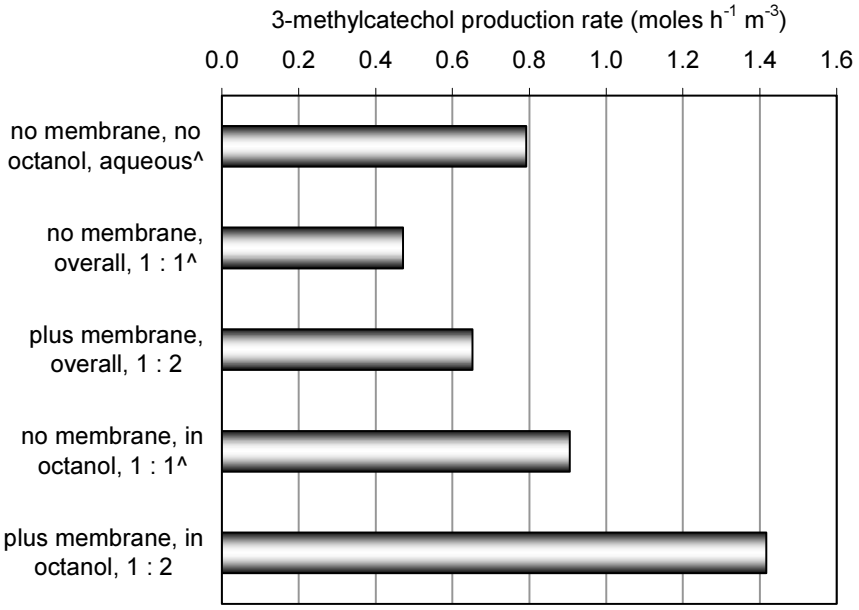

Figure 2 Comparison of overall 3-methylcatechol production rates. ^ Calculated from Hüsken *et al.*, 2001b.

Downstream processing of 3-methylcatechol from octanol

The octanol phase contained some extracted LB medium components, the substrate toluene and the majority of the product 3-methylcatechol. Purification steps are required to obtain the product.

The boiling point of 3-methylcatechol is 241 °C, while octanol has a boiling point of 195 °C and toluene of 111 °C at atmospheric pressure. The product thus cannot be evaporated from the organic solvent. To evaporate toluene and octanol requires much energy and the remaining product would contain

significant quantities of concentrated impurities from the culture medium. Safety is an issue as well (Schmid *et al.*, 1999), since both toluene and octanol are inflammable and explosive with air.

There are several alternative ways to recover the product from the organic phase. Product recovery by adsorption is a possibility (Held *et al.*, 1998; Robinson *et al.*, 1992), although competition between toluene and 3-methylcatechol for binding onto the adsorbent phase may limit the efficiency of this method. In this research, we extracted the product into an alkaline aqueous phase (1.0 M sodium hydroxide) for further downstream processing. A solution of 1.0 M of sodium hydroxide can be used to deprotonate 3-methylcatechol and to extract its disodium salt into the aqueous alkaline solution (Hüsken *et al.*, 2001b). This solution should be separated from the organic phase, which can be recycled to the production process. The alkaline solution should then be acidified to reprotonate 3-methylcatechol, which can subsequently be recovered by extraction into another solvent in which the product dissolves well and that is more volatile than octanol, for example dichloromethane. Subsequent phase separation and solvent evaporation will then yield 3-methylcatechol (Figure 3).

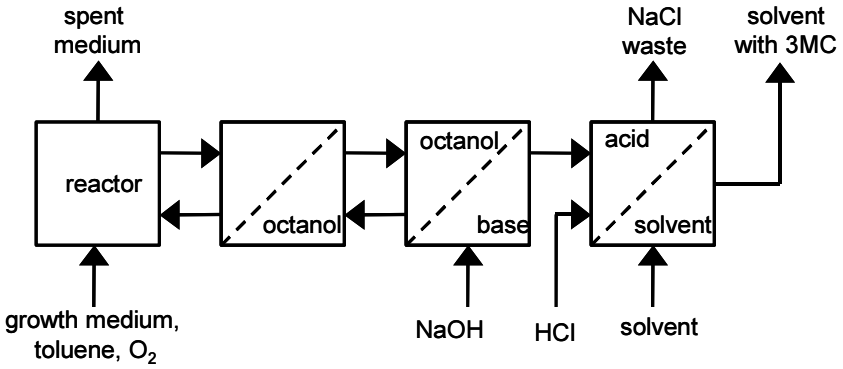

Figure 3 Schematic representation of possible process, including downstream processing.

An additional advantage of sodium hydroxide is that the alkaline phase with the product will probably not contain apolar impurities. After further extraction

of 3-methylcatechol from this aqueous phase into a different organic phase, the product phase will also not contain polar impurities, which remain in the salt solution.

The extraction capacity of 1.0 M NaOH was measured at different fractions of NaOH solution and octanol and at different contact times (Figure 4A, B). At the start, the octanol was transparent, slightly light brown and contained 60 mM of 3-methylcatechol and 400 mM of toluene. Upon addition of this solution to 1.0 M NaOH, the interface turned black; after mixing, a very dark green, almost black emulsion was obtained. These emulsions were shortly centrifuged to separate the phases. After acidification of the alkaline phase (Arnow, 1937), the aqueous 3-methylcatechol concentration was measured. If samples were not analysed immediately, lower product concentrations were measured. This was anticipated, since 3-methylcatechol is not stable at high pH (Hüsken *et al.*, 2001a; Robinson *et al.*, 1992). A fast extraction process is therefore needed.

As expected, the 3-methylcatechol concentration found in NaOH was highest at the lowest volume fraction of NaOH solution (287 mM of the sodium salt of 3-methylcatechol; Figure 4A). The recovery after one minute (57% ± 3.0 (standard deviation)) does not differ much for different volume fractions of NaOH (Figure 4B). At first, we found this surprising, since one would expect a higher recovery at higher alkaline fractions. However, if equilibrium is not reached and if salt formation is rate-limiting and/or a constant emulsion surface area is present, then a constant recovery is expected. Besides that, instability of the product at high pH is an issue and will influence the recovery (Figure 4B). It is thus advisable to apply a short contact time and to use only a small fraction of NaOH, which contains high concentrations of the product and therewith facilitates downstream processing.

If only one extraction step is applied, octanol still contains a large amount of 3-methylcatechol. Additional extraction steps will obviously result in a higher recovery, but a 100% recovery is not achievable due to product degradation in the alkaline phase. A continuous counter-current extraction would be better, provided residence times are kept low. Because of the use of the membrane, biomass is not present and can thus not interfere with the extraction process.

Furthermore, toluene hardly dissolves in the aqueous alkaline phase (Hüsken *et al.*, 2001b) and can be recycled together with the octanol phase. Although this is not the only alternative, for the selective extraction of 3-methylcatechol from octanol, the use of NaOH (Figure 4) is a useful option.

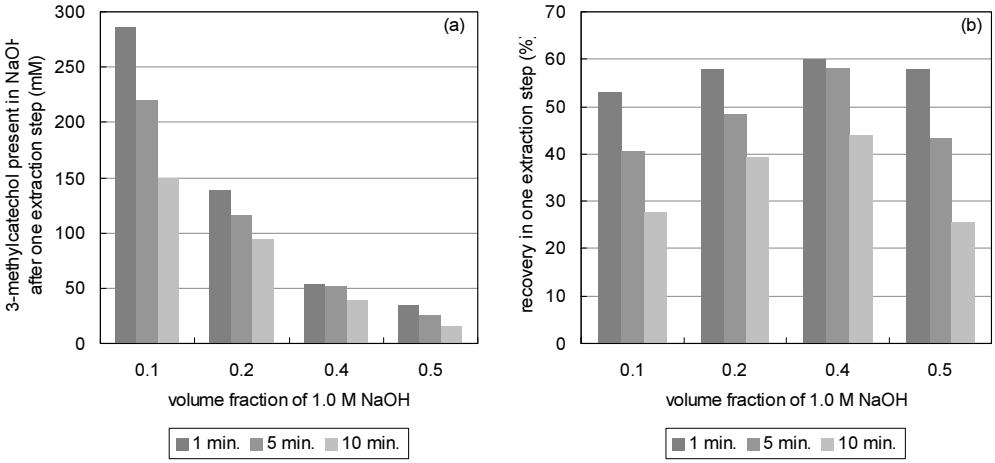

Figure 4 Extraction of 3-methylcatechol from octanol in 1.0 M sodium hydroxide. (a): concentration of 3-methylcatechol present in NaOH after one extraction step. Initially present in octanol: 60 mM of 3-methylcatechol. (b): recovery of 3-methylcatechol (% of total product in NaOH) in one extraction step. Contact times 1, 5 and 10 minutes.

Bioproduction and recovery of 3-methylcatechol: membrane feasibility

The use of a membrane in organic/aqueous two-phase bioreactors was previously described for enzymatic and microbial conversions (Shin *et al.*, 2001; Gerigk *et al.*, 2000; Hoogakker *et al.*, 2000; Choi *et al.*, 1999; Molinari *et al.*, 1997). Choi *et al.* (1999, 2000) used a hydrophilic membrane bioreactor, preventing direct contact between yeast cells and the organic solvent. A hydrophobic membrane was found to be less appropriate (Choi *et al.*, 1999); this was attributed to phase toxicity inside the membrane pores. Since the specific growth rate we found in single-phase aqueous reactions (Hüsken *et al.*, 2001b) was basically the same as in the membrane set up (Figure 1), phase toxicity inside membrane pores apparently was of no consequence in our study.

When we used a hydrophilic hollow-fibre membrane (results not shown), a slightly higher pressure on the octanol phase was applied, to prevent permeation of the aqueous phase. Besides that, the aqueous phase had to be pumped at high speed to return the cells to the bioreactor, to prevent oxygen depletion. This specific hydrophilic membrane module was incapable of keeping both phases separated. It is expected that this was a result of the diameter of the fibres (0.1 mm), that caused high pressure drops under the applied conditions. The hydrophobic membrane (fibre diameter 1.8 mm) that was ultimately used did prove to be suitable for our bioprocess; it prevented mixing of phases and emulsification.

When comparing the membrane reactor with e.g. emulsion reactors, not only the productivity of the reactors (described in these results) should be considered, but also other effects come into play, such as the formation of emulsions and contamination of the organic phase with cell material. Jauregi *et al.* (2001) described interfacial partitioning of bioparticles in alkanol/water systems; their interface was a thick, emulsion-like layer that was stabilised by the presence of crystals (Pickering stabilisation). Also in the absence of crystals, octanol/aqueous emulsions are quite stable and difficult to separate (Schroën and Woodley, 1997). In one of our studies in which we allowed direct contact, we found that, after sedimentation, an inter-phase was formed that proved difficult to separate. In most cases, the inter-phase is considered a loss and therewith the overall productivity decreases. If direct phase contact is allowed, the octanol phase will be contaminated with cells and cell debris that affect the efficiency of downstream processing (Mathys *et al.*, 1998a, 1998b). In the membrane reactor this direct contact is prevented, and therefore, downstream processing will be facilitated and overall productivity will be further improved.

It is thus clear that application of the membrane improved the process in various ways. The octanol phase that contained the bulk of the product remained separated from the culture medium and higher volumetric production rates based on octanol were found (Figure 2), compared to processes without a membrane (Hüsken *et al.*, 2001b). Besides that, emulsion formation and contamination of the octanol phase with cells and cell debris was prevented,

giving rise to additional advantages. The volumetric production rate can even be further improved if transport of the product through the membrane is enhanced by either lowering the membrane resistance or increasing the membrane surface area. In conclusion, the effects described here are very general, and the membrane can be used to improve any other organic/aqueous bioconversion that suffers from e.g. emulsion formation.

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Chapter 6

Model description of bacterial 3-methylcatechol production in two-phase systems

ABSTRACT

Pseudomonas putida MC2 produces 3-methylcatechol from toluene in aqueous medium. The presence of a second phase of 1-octanol improves product accumulation. To obtain a good design and to be able to optimise this process, a process model was developed, both for one- and two-phase applications. The insights obtained by the model predictions showed the importance of different process parameters on growth of biomass, accumulation of 3-methylcatechol and process time. For future applications, the process model can be used to ensure enough extraction capacity from aqueous to octanol phase. It is a useful tool to define the optimum process conditions, depending on the desired optimisation parameter: product concentration or processing time.

This chapter was submitted for publication (LE Hüsken, J Hoogakker, JAM de Bont, J Tramper, HH Beeftink (2002))

INTRODUCTION

Substituted catechols are valuable precursors in pharmaceutical production processes (Ennis and Ghazal, 1992; Van Scharrenburg and Frankena, 1996), but they are difficult to synthesize chemically (Held *et al.*, 1999; Van Scharrenburg and Frankena, 1996). We studied the model conversion of toluene to 3-methylcatechol by *Pseudomonas putida* MC2 in aqueous media (Hüsken *et al.*, 2001a, 2002a). Growing cells are needed for this bioconversion, but growth stops early due to elevated concentrations of the inhibitory product.

Production levels and rates may be enhanced by introduction of a second, organic phase that provides the substrate and extracts the product from the culture medium. 1-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 (Hüsken *et al.*, 2001b).

Insight in the process is imperative for a rational design. A reliable description of growth of *P. putida* MC2 and production of 3-methylcatechol is required. Modelling of bioproduction processes in organic/aqueous two-phase systems has been described before, for example for the enzymatic synthesis of (*R*)-mandelonitrile (Willeman *et al.*, 2001, 2002) and the enzymatic hydroxylation of phenol (Chae and Yoo, 1997). Cesário *et al.* (1997) described modelling and validation of a dispersed organic solvent in a three-phase (organic/aqueous/gaseous) system for the biological treatment of waste gases. We developed a model to predict and optimise bacterial growth and 3-methylcatechol production in both one- and two-phase systems. To our knowledge, not much has been published on modelling of these kinds of bacterial production processes.

A process model was developed for 3-methylcatechol production in biphasic fed-batch mode. It consists of mass balances over the aqueous and the organic phase. It predicts the aqueous and organic 3-methylcatechol concentrations, the viable, active biomass concentration, and the concentration of the growth substrate.

In the aqueous phase, growth and production take place. High product concentrations inhibit growth of the bacterium (Robinson *et al.*, 1992; Hüsken *et al.*, 2001b). The product 3-methylcatechol partitions favourably from the aqueous to the octanol phase. These presumptions define the model conditions. The process model describes our results satisfactory, as we will show in this paper, and it can be used to improve process conditions in order to achieve a more advantageous result.

MATERIALS AND METHODS

Biocatalyst strain, growth, and process conditions

Pseudomonas putida MC2 (Hüsken *et al.*, 2001a) accumulates 3-methylcatechol. Media, induction and process conditions for one- and two-phase systems are similar to those previously described by Hüsken *et al.* (2001b). A schematic overview of the process is given in Figure 1.

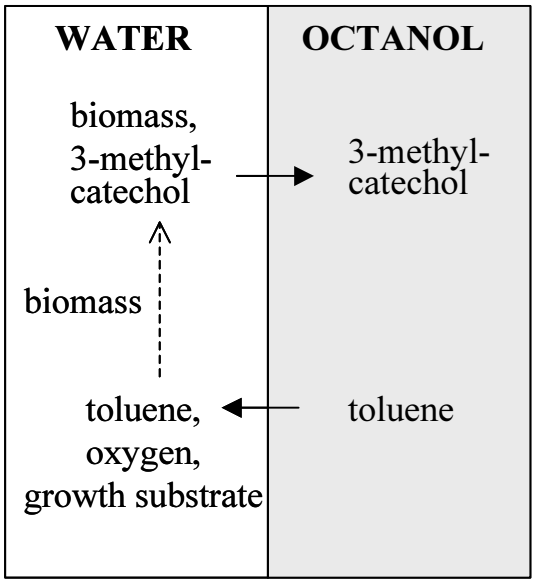

Figure 1 Schematic representation of the production and extraction process.

3-Methylcatechol analysis

Determination of 3-methylcatechol concentrations in the aqueous phase was done using Arnow's method (Arnow, 1937). For the octanol phase, Arnow's method was applied as described by Hüsken *et al.* (2001b); partitioning of 3-methylcatechol over octanol and LB medium (Sambrook *et al.*, 1989) was 22 on a volume basis.

Stability of 3-methylcatechol was measured in 250-ml airtight bottles that contained 10 ml of a medium buffer at pH 7. Different concentrations (1, 4, 10 and 20 mM) of 3-methylcatechol were dissolved in the buffer and bottles were incubated in a shaking waterbath at 30°C. The 3-methylcatechol concentration was monitored in time, to define a parameter that would describe how fast the product is polymerised or oxidised.

Mass transfer

Toluene is supplied via octanol that contained 400 mM of toluene at time $t = 0$, which is in equilibrium with 1.0 mM of toluene in LB (Hüsken *et al.*, 2001b); in addition, toluene is supplied via the incoming gas stream, which contained 0.26 mM of toluene, which also is in equilibrium with 1.0 mM in water (Amoore and Hautala, 1983). This latter concentration in LB is assumed to be constant during the process (supply is faster than degradation) and mass transfer of toluene is thus not taken into account in the model.

The surface area A between LB and octanol cannot easily be measured and the value of mass transfer coefficient k was also not known, but they can be estimated and their effect on mass transfer of 3-methylcatechol can be studied with the model.

Model assumptions

Based on previous results, the following assumptions were applied. Growth of *P. putida* MC2 and production of 3-methylcatechol take place in the aqueous phase only. The oxygen concentration in the aqueous medium is assumed not

limiting the process and is therefore not a model variable. The product dissolves better in octanol than in the aqueous medium. Octanol initially contains the substrate toluene but no product. Volumetric influence of concentrated growth substrate addition (feed rate F_{in}, concentration C_{sin}) and the extraction of samples on the volumes of both phases in the reactor are assumed to be negligible. The volumes of the aqueous (V_w) and the organic (V_{oct}) phases stay constant during the process. Growth and production are limited by the product 3-methylcatechol (concentration C_{pw}, inhibition factor K_{ip}) in the aqueous phase. A lag time (t_{lag}) is introduced, because in organic/aqueous two-phase systems a long lag phase was found during which growth and production was nihil (Hüsken *et al.*, 2001b). In aqueous one-phase systems, the lag time was zero. The concentration of inducer salicylate for the reaction (Hüsken *et al.*, 2001a) is constant and not taken into account in the model.

Nomenclature is given in Table 1. Balances are provided in the following section.

Process model

The model predicts:

1. the viable, active biomass concentration C_x,
2. the concentration of the growth substrate C_s,
3. the aqueous 3-methylcatechol concentration C_{pw},
4. and the organic 3-methylcatechol concentration C_{poct},

using four equations.

The equations are valid for $t > t_{lag}$. If $t \leq t_{lag}$, the differential equations equal zero and $C_x = C_x(0)$, $C_s = C_s(0)$, $C_{pw} = C_{pw}(0)$ and $C_{poct} = C_{poct}(0)$. All parameters are given in Table 1.

Table 1 Process model parameters: nomenclature and value(s) used. 1P = one phase, 2P = two-phase process.

Parameter	Definition	Value
A	surface area between octanol and LB	0 m^2 (1P); variable (2P)
C_{pw}	product concentration in aqueous phase	variable, $mol \cdot m^{-3}$
C_{poct}	product concentration in octanol phase ($22 \cdot C_{pw}$)	variable, $mol \cdot m^{-3}$
$C_s(0)$	initial growth substrate concentration	89 $mol \cdot m^{-3}$
$C_s(in)$	feed growth substrate concentration	1335 $mol \cdot m^{-3}$
C_x	biomass concentration	1.64 mM[#]
F_{in}	ingoing feed rate	$2 \cdot 10^{-6}$ $m^{-3} \cdot h^{-1}$
k	mass transfer coefficient of product from LB to octanol	0 $m \cdot h^{-1}$(1P); 0.05 $m \cdot h^{-1}$ (2P)
K_{cd}	fit parameter to make k_d exponentially dependent on C_{pw}	1.4 $mol \cdot m^{-3}$ (1P); 0.24 $mol \cdot m^{-3}$ (2P)
k_d	death rate constant	$7.0 \cdot 10^{-4}$ h^{-1} (1P); $2.1 \cdot 10^{-5}$ h^{-1} (2P)
K_{ip}	growth inhibition constant by product	1.9 $mol \cdot m^{-3}$
K_P	first-order polymerisation constant	0.004 h^{-1}
K_s	Monod constant	10 $mol \cdot m^{-3}$
m	partition coefficient of product over octanol and LB medium[‡]	22 m^3 LB$\cdot m^{-3}$ oct
m_s	maintenance coefficient[¶]	$1.81 \cdot 10^{-3}$ $mol \cdot mol^{-1} \cdot h^{-1}$
t_{lag}	lag time for growth and bioproduction	0 h (1P); 20 h (2P, 3 : 2)
q_p	specific 3-methylcatechol production rate[‡]	0.0244 $mol \cdot mol^{-1} \cdot h^{-1}$
V_{oct}	octanol volume	0 m^3 (1P); variable (2P)
V_w	aqueous (LB) volume	$0.8 \cdot 10^{-3}$ m^3 (1P); variable (2P)
Y_{xs}	yield of biomass on growth substrate[¶]	0.66 $mol \cdot mol^{-1}$, [#]
μ_{max}	maximum specific growth rate	1.05 h^{-1}

[#] based on C-moles; initial value

[‡] Hüsken *et al.*, 2001b; [¶] Van 't Riet and Tramper, 1991

The biomass equation reflects 3-methylcatechol-inhibited growth and 3-methylcatechol-stimulated death. The death rate of the cells was subtracted from the specific growth rate to adequately describe production by growing or viable cells (Van 't Riet and Tramper, 1991). A first-order death rate constant k_d was thus introduced, together with a fit parameter K_{cd} to make k_d increase exponentially with the concentration of 3-methylcatechol in water. Growth inhibition by toluene was negligible at the low toluene concentration applied in the culture medium (Hüsken *et al.*, 2001b) and is therefore not taken into account in the equation. The biomass concentration C_x in the aqueous phase in time is then given by:

$$\frac{\mathrm{d}C_x}{\mathrm{d}(t - t_{lag})} = r_x = \left(\mu_{max} \cdot \frac{C_s}{K_s + C_s} \cdot \mathrm{e}^{-\frac{C_{pw}}{K_{ip}}} - k_d \cdot \mathrm{e}^{\frac{C_{pw}}{K_{cd}}} \right) \cdot C_x \qquad (\mathrm{mol \cdot m^{-3} \cdot h^{-1}}) \ (\mathbf{1})$$

The concentration of growth substrate in the aqueous medium is defined by the use for growth and maintenance of the cells and the feed addition. The growth substrate concentration C_s in the aqueous phase in time is given by equation (**2**):

$$\frac{\mathrm{d}C_s}{\mathrm{d}(t - t_{lag})} = -\left(\frac{\mu_{max}}{Y_{xs}} \cdot \frac{C_s}{K_s + C_s} \cdot \mathrm{e}^{-\frac{C_{pw}}{K_{ip}}} + m_s \right) \cdot C_x + F_{in} \cdot C_{sin} \qquad (\mathrm{mol \cdot m^{-3} \cdot h^{-1}}) \ (\mathbf{2})$$

Production of 3-methylcatechol by the bacterium, as well as transport of the product from the aqueous to the organic phase, define the mass balance of 3-methylcatechol in the culture medium. Furthermore, stability of the product is an issue. At physiological pH, 3-methylcatechol is not completely stable; therefore a degradation term was inserted in equation (**3**). For loss of product, only chemical degradation is taken into account, because biological degradation of 3-methylcatechol by *P. putida* MC2 is not possible (Hüsken *et al.*, 2001a). In the case of a one-phase system, the surface area between

culture medium and octanol, A, equals zero and the transport term in equation (**3**) disappears. This also applies for equation (**4**).

The concentration of 3-methylcatechol in the aqueous phase C_{pw} in time is thus given by:

$$\frac{dC_{pw}}{d(t-t_{lag})} = q_p \cdot C_x - k_p \cdot C_{pw} - \frac{k \cdot A}{V_w} \cdot \left(C_{pw} - \frac{C_{poct}}{m} \right) \qquad (mol \cdot m^{-3} \cdot h^{-1}) \quad (\mathbf{3})$$

Presence of 3-methylcatechol in octanol is entirely due to transport from the aqueous phase. The concentration of 3-methylcatechol in the octanol phase C_{poct} in time is given by equation (**4**):

$$\frac{dC_{poct}}{d(t-t_{lag})} = \frac{k \cdot A}{V_{oct}} \cdot \left(C_{pw} - \frac{C_{poct}}{m} \right) \qquad (mol \cdot m^{-3} \cdot h^{-1}) \quad (\mathbf{4})$$

These four equations present the model for growth of *Pseudomonas putida* MC2 and bioproduction of 3-methylcatechol in one- and two-phase systems. This model was programmed in Mathcad 2000 Professional.

RESULTS AND DISCUSSION

Parameters

All parameters and values used in the process model are given in Table 1.

Toluene at an aqueous concentration of 1.0 mM hardly affected the bacterium, as was previously reported (Hüsken *et al.*, 2001b). An inhibitory constant for toluene was thus not implemented. The inhibition constant of 3-methylcatechol K_{ip} was measured in a simple growth experiment at four different 3-methylcatechol concentrations, yielding K_{ip} = 1.9 mol·m^{-3} (data not shown). For aerobic growth, a common yield of biomass on growth substrate

Y_{xs} is 0.55 g·g^{-1} (Van 't Riet and Tramper, 1991), corresponding to 0.66 mol·mol^{-1} (based on C-moles).

The specific production rate q_p in equation (**3**) was calculated from previous results (Hüsken *et al.*, 2001b). First-order polymerisation constant k_p was measured in a buffer at pH 7 and 30 °C. All initial concentrations (1, 4, 10 and 20 mM) showed a product decay of approximately 20 % in 50 hours, corresponding to k_p = 0.004 h^{-1} (equation (**3**)). At pH 7 and on this time scale, loss of product caused by chemical instability will thus be limited.

The growth medium LB was a complex, rich medium. Growth on LB medium was equivalent, with respect to final biomass concentrations, to growth on a defined medium of 89 mM of succinate. We therefore used this value of 89 mM for modelling purposes ($C_s(0)$ = 89 mM). Feed of growth substrate to the fermentor, starting at time t = 0, contained 15 times concentrated LB medium, with respect to nutritional compounds (corresponding to 1335 mM of succinate) and was provided at a constant feed rate F_{in} of 2 ml h^{-1}.

Parameter K_{cd} in the model formed a product-dependent death rate of the cells (equation (**1**)). If the one-phase values of k_d and K_{cd} were used in the biphasic process, inactivation of biomass was too slow and product concentrations rose to non-realistic values. Octanol affected the death rate, or at least the activity of the cells. Values of k_d and K_{cd} were therefore different for one- and two-phase systems (Table 1).

One-phase modelling

Figure 2 shows results of one-phase growth of *P. putida* MC2 and bioproduction of 3-methylcatechol. Production stopped after 12 hours, when 10 mM of 3-methylcatechol was produced. The model describes our data well.

In the model, only active biomass is taken into account. The model shows an optimum in its concentration; it then decreases to zero, because cells die due to the high product concentration. The maximum active biomass concentration, according to the model, was 1.4 g l^{-1}, or 56 mM (based on C-

moles of biomass). Experimental data show the total concentration of biomass, including non-viable or dead cells (Figure 2).

The growth substrate concentration (not measured) decreased from 89 mM (t = 0) to 2 mM (t = 10 h). Increasing the feed rate or feed substrate concentration, both in the model and in experiments, had a small effect on growth and 3-methylcatechol production: doubling $C_s(0)$ and $C_s(in)$ resulted in a $C_x(max)$ of 1.7 instead of 1.4 $g \cdot l^{-1}$ and a $C_{pw}(max)$ of 10.6 instead of 10.1 mM. These maximum concentrations were produced in the same process time and at the end of this process with doubled C_s, 65 mM of growth substrate was still present. The process was thus only slightly limited by C_s in the model. The end of the process was caused by inhibition of *P. putida* MC2 by the increased level of 3-methylcatechol.

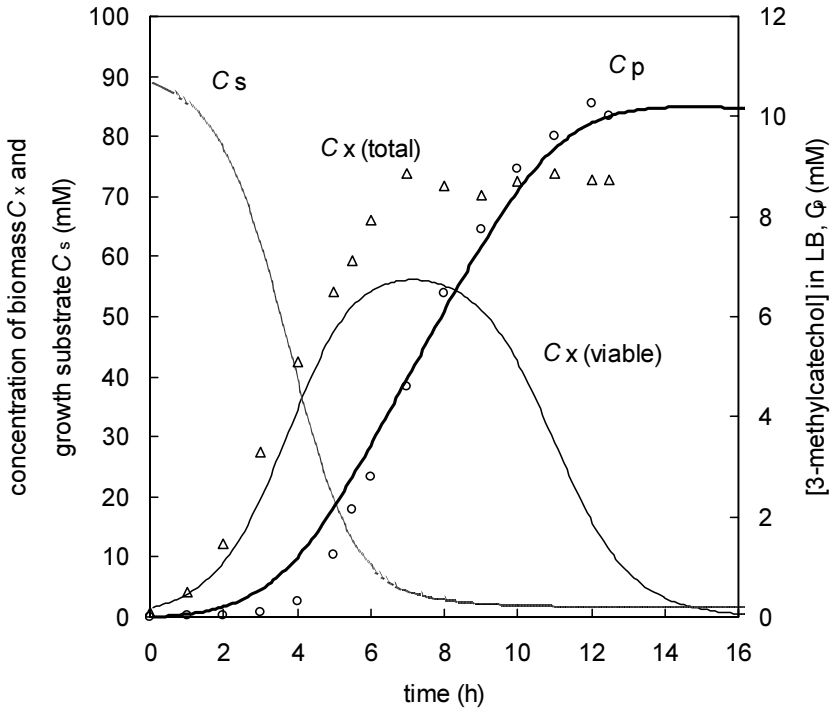

Figure 2 Model simulation and experimental data of biomass and product concentrations in a one-phase experiment without octanol. Measured biomass (Δ) and product (○) concentrations are derived from Hüsken *et al.* (2001b); C_x in the model predictions depicts only active biomass, which explains the difference with the measured C_x values.

We were satisfied with the model predictions for one-phase growth and bioproduction of 3-methylcatechol (Figure 2) and used the same model for the two-phase process.

Two-phase modelling

In Figure 3 biomass and growth substrate concentration in LB, and 3-methylcatechol accumulation in octanol and in LB are depicted for a two-phase process with a phase ratio of 2 (octanol) : 3 (LB). See also Table 1 (V_{oct} = 320 ml, V_w = 480 ml).

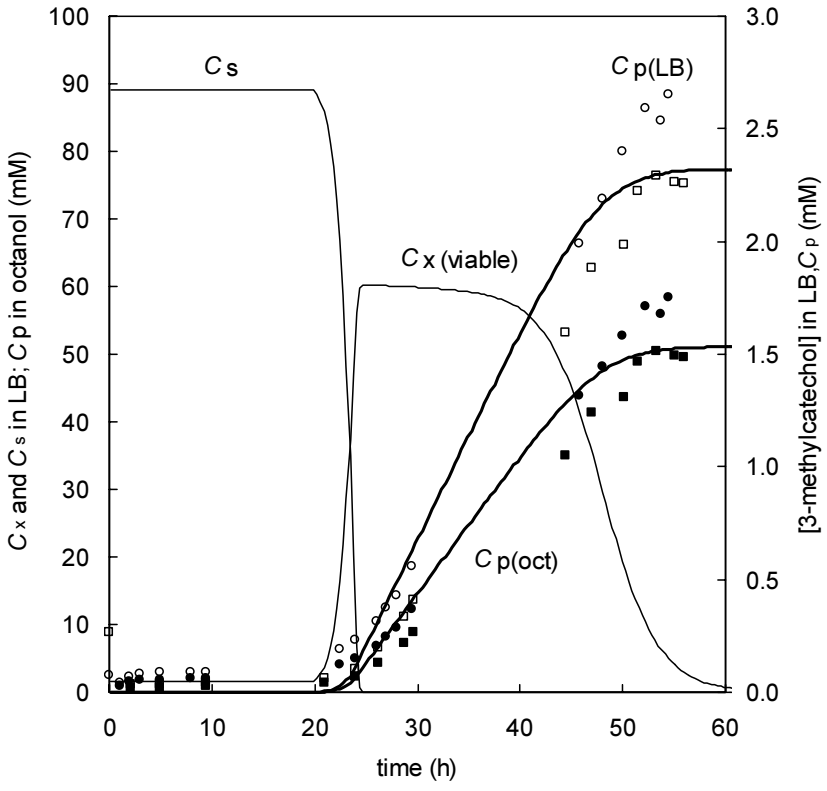

Figure 3 Model simulation and experimental data of product concentrations in two-phase experiments (duplo) with direct phase contact. Phase ratio octanol : LB (aqueous) = 2 : 3. Product concentrations (○, ●, □, ■; open symbols are concentrations in aqueous phase) are from Hüsken *et al.* (2001b). Biomass was not measured in two-phase systems with direct phase contact. The biomass graph depicts only active biomass.

Production started after cells had adapted to the fermentation conditions (the presence of a second phase of octanol). After approximately 53 hours, when not much viable biomass was left (Figure 3), production of 3-methylcatechol ended at an aqueous concentration of 2.3 mM, corresponding to 51 mM in octanol. The model describes the data satisfactory (Figure 3).

Our data showed that the two-phase process took longer than the one-phase process (Figure 2). Cells that entered the reactor at time t = 0 hours were active, but when octanol was added, many died and the surviving cells needed time to adapt and to grow. A lag time of 20 hours was applied in this model (phase ratio 2 : 3), since there was no clear sign of activity (low CO_2 production (results not shown) and no product accumulation (Figure 3)) during this period.

The growth substrate concentration C_s decreased rapidly during growth (Figure 3). The maximum biomass concentration in the two-phase process was 1.5 g l^{-1}, reached after 26 hours, as predicted by the model. It was not possible to quantitatively measure biomass concentrations in this two-phase system with direct phase contact (Hüsken *et al.*, 2001b). Cells stayed active and produced 3-methylcatechol at an almost constant rate and after approximately 40 hours, according to the model, cells started to die (Figure 3).

It was checked if the low growth substrate concentration caused the end of the process. Upon doubling the substrate concentrations $C_s(0)$ and $C_s(in)$ in the model, growth was improved: C_x was 2.9 instead of 1.5 g l^{-1}. Product levels however hardly increased: C_{pw} was 2.5 instead of 2.3 mM and C_{poct} 55 instead of 51 mM. The process time decreased from 53 to 40 hours. Extra growth substrate was clearly used to produce more biomass and since these cells were actively producing 3-methylcatechol, the process was faster. High C_x thus resulted in faster production of 3-methylcatechol (equation (**3**)), but this did not imply a larger final product concentration: accumulation of toxic product under these aqueous conditions (in the direct presence of a second phase of octanol), and not the limited availability of C_s, probably caused the end of the process.

Comparison of processes with different phase ratios

In Table 2, one- and two-phase processes are compared with respect to maximum biomass and product concentrations at different phase ratios. Overall averaged 3-methylcatechol concentrations from experiments (Hüsken *et al.*, 2001b) and from the model are given in Figure 4. The model was not only valid for the examples shown in Figures 2 and 3, but also for other phase ratios (Figure 4).

Table 2 Effect of phase ratio on final biomass and product concentrations, according to the process model and to experiments. Total volume of both phases is 0.80 litre.

Phase ratio (oct : LB)	Model			Experiment [∂]	
	C_x (g·l^{-1})	C_{poct} (mM)	C_{pw} (mM)	C_{poct} (mM)	C_{pw} (mM)
0 : 10	1.4	0	10	0	9.5 (± 1.9) [§]
1 : 4	1.5	56	2.5	65	3.0 (± 0.35)
2 : 3	1.5	51	2.3	54	2.5 (± 0.25)
1 : 1	1.5	49	2.2	48	2.2 (± 0.28)
4 : 1	1.5	41	1.9	-	-

[∂] Data from Hüsken *et al.* (2001b). Standard deviation is shown in brackets
[§] Maximum measured C_x in one-phase experiment: 1.8 g·l^{-1}

Only at low octanol fractions the model prediction differed from the measured data, caused by the fact that only the two-phase model values (Table 1) are used in Figure 4. This resulted in a deviation near zero octanol, whereas the one-phase model did predict the correct concentration of 10 mM (Figure 2).

Growth in two-phase systems stopped at a 3-methylcatechol concentration between 0.2 – 1 mM, depending on phase ratio and growth substrate concentration. In all cases, the growth substrate was practically depleted. The feed of concentrated LB medium was directly consumed. Death of the cells could be fast and immediately (1 (octanol) : 4 (LB)), or slow (1 : 1, 4 : 1), depending on the aqueous product concentration. The presence of a second phase of octanol in two-phase systems caused extra stress for the biomass. In

one-phase systems, growth stopped at a product concentration of 5 mM (Figure 2).

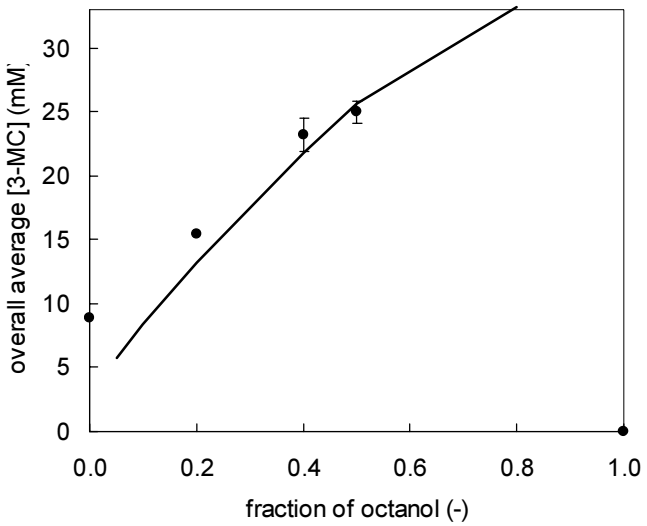

Figure 4 Comparison of averaged 3-methylcatechol concentrations over LB medium and octanol together (●) (Hüsken *et al.*, 2001b) and the model prediction (_).

Process times are not taken into account in Table 2. The higher the octanol fraction in the process was, the larger the process time became. For example, at 80% of octanol (octanol : LB = 4 : 1), the model predicted a process time of 200 hours. The biomass growth rate was similar to the process in Figure 3, but cell death was much slower due to the low aqueous product concentration, making the process much slower, which is why we did not test these conditions (4 : 1) in the reactor. This finding of an extremely high process time at large octanol fractions was confirmed in small-scale experiments (Hüsken *et al.*, 2001b).

Previously (Hüsken *et al.*, 2002b), we studied a two-phase system without direct contact between octanol and the culture medium, by means of a membrane barrier. No lag time was found and more product accumulated in the aqueous phase, now that phase toxicity (Bar, 1988) was overcome. The process time was reduced (Hüsken *et al.*, 2002b) and comparable to one-phase production.

Sensitivity analysis

The influence of some parameters on the outcome of the model was tested. The initial biomass concentration, the growth substrate concentration, the 3-methylcatechol partition coefficient and the mass transfer rate were altered to observe their effects on the model predictions. Results are shown in Figure 5. As a standard, the conditions from Figures 2 and 3 were used, corresponding to coordinates (1, 1) in Figure 5.

The initial biomass concentration hardly affected the final C_x, nor did it affect accumulation of product or the process time, in one- and two-phase models (Figure 5A). The growth substrate concentration ($C_s(0)$ and $C_s(in)$) had a larger impact, especially on growth and on the process time (Figure 5B). Doubling C_s resulted in a decrease of the process time with 14% (one-phase) to 27% (two-phase), while biomass increased by 26% to 97%, respectively. Product accumulation was slightly affected (+4% in one-phase, +7% in two-phase process upon doubling C_s) (Figure 5B).

In Figure 5C, the effects of the partition coefficient m of 3-methylcatechol over octanol and culture medium were studied. In experiments, this would imply the use of a different medium. A higher product affinity for an organic solvent other than octanol for example, would result in an elevated partition coefficient. As expected, an elevated m resulted in a higher C_{poct} and a somewhat lower C_{pw} as compared to the standard conditions. Biomass growth rate and maximum C_x were not affected (Figure 5C), while the process time increased, because the aqueous product concentration rose slower and thus toxicity (causing cell inactivity) was postponed. More product could therefore accumulate at higher m. Nevertheless, octanol cannot simply be replaced by a solvent that would yield better partitioning of 3-methylcatechol. Toxicity of the solvent towards the biocatalyst determines its potential and would have to be investigated first. This will influence growth, process time and production.

Mass transfer over the octanol/aqueous interface was studied by altering $k{\cdot}A$. According to the model, equilibrium of the product over the two phases exists (Figure 5D). The values used under standard conditions were not rate-limiting. To see an effect on mass transfer, $k{\cdot}A$ must be lowered considerably

(Figure 5D): 3-methylcatechol concentrations in both phases dropped 2% at $k{\cdot}A{\cdot}0.1$, while at $k{\cdot}A{\cdot}0.02$, 11% less product accumulated in both phases. Slower transport would result in a situation were production becomes faster than extraction to octanol.

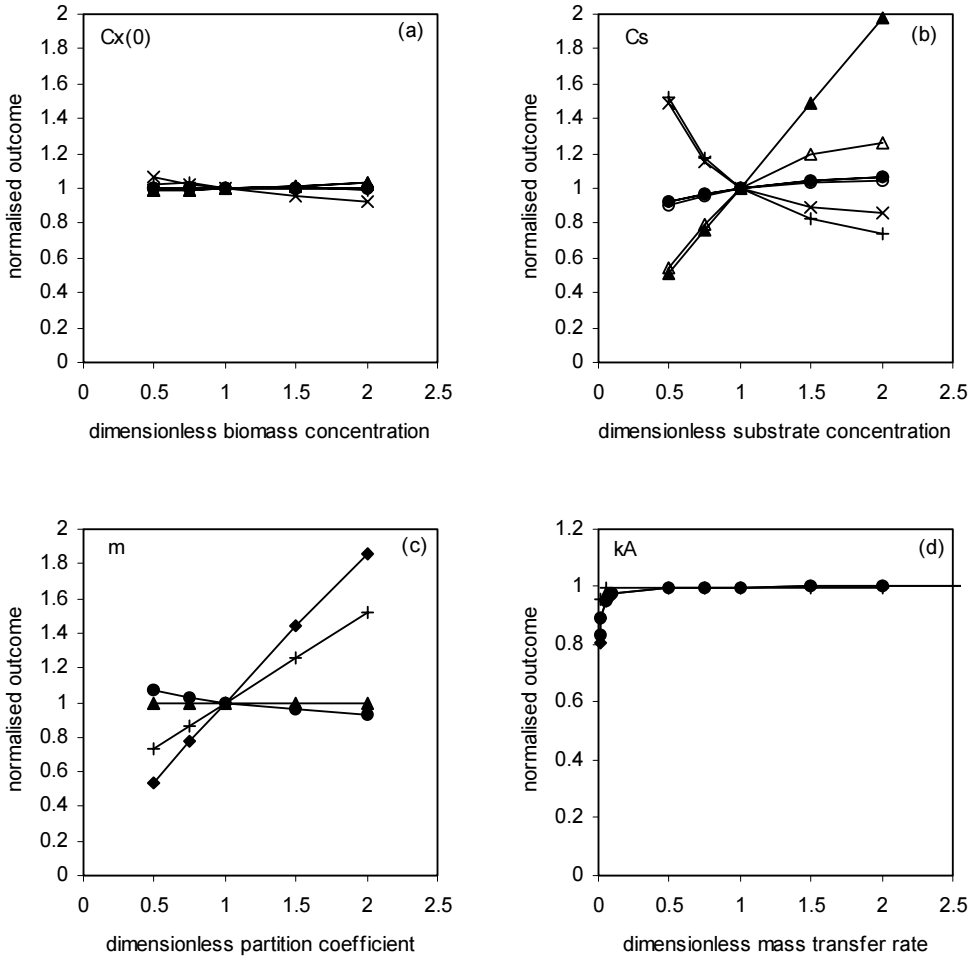

Figure 5 Normalised dimensionless parameters and a normalised dimensionless model outcome for one-phase (open symbols) and two-phase (filled symbols) processes. The effect of the value of parameters $C_x(0)$ (a), C_s (b), m (c) and $k{\cdot}A$ (d) on the outcome of the model is depicted, with respect to $C_x(\max)$ (Δ, ▲), $C_{pw}(\max)$ (○, ●), $C_{poct}(\max)$ (♦) and process time (time to achieve $C_{pw}(\max)$); × for one phase, + for two phases).

Outlook

In previous results (Hüsken *et al.*, 2002b), production was in fact faster than extraction and thus no equilibrium existed over both phases. Since in the latter process, a membrane was used to keep the phases separated, the surface area for transport of 3-methylcatechol (A) is correlated to the membrane area. By using the model, we can see how mass transfer was influenced quantitatively and what was needed for equilibrium. In other words, the model shows how large the area for mass transfer (A) or how small the transport resistance (~1/k) should have been to obtain a process with faster extraction than production (Hüsken *et al.*, 2002b).

For future applications, the process model can be used to ensure enough extraction capacity in terms of $k{\cdot}A$. The model on growth of *P. putida* MC2 and bioproduction of 3-methylcatechol in the presence and absence of a second organic solvent gives more insights into the process. Concluding from our results, the growth substrate concentration should be elevated to decrease the process time, while slightly increasing production. The model provides a useful tool to define the optimum process conditions, depending on the desired optimisation parameter: product concentration or process time.

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Chapter 7

General discussion

Biocatalysis applies the catalytic potential of enzymes to produce building blocks and end products for the pharmaceutical and chemical industry (Held *et al.*, 2000). Scientific progress in this area has resulted in a wide range of industrial applications, while in the mean time industries worldwide acknowledge the growing importance of sustainable production processes.

OBJECTIVE

The objective of the study described in this thesis was to optimise biocatalytic production of catechol by an integrative approach, including both microbiological and process-engineering aspects. Since catechols are toxic for many micro-organisms (Chapter 1), we studied the microbial production of these compounds and investigated ways to deal with product toxicity and to optimise the bioproduction process.

In this chapter, we will shortly discuss the obtained 3-methylcatechol production rates. Subsequently, we will consider (alternative) catechol bioproduction processes. Finally, industrial relevance and concluding remarks are presented.

3-METHYLCATECHOL PRODUCTION RATES: COMPARISON OF PROCESSES

3-Methylcatechol production rates were found to depend on the type of growth substrate, on pH and on the toluene concentration. Depending on the

optimisation criterion (specific production rate, volumetric production rate, downstream costs), a certain set of process conditions had to be chosen for optimal production (Chapter 3).

Specific production rates

In this particular process, activity of the biomass is not truly rate-limiting. The specific catechol production rate is no decisive factor from a technological or economical point of view, but gives more insight into the behaviour of bacteria. The challenge here lies in process engineering.

Microbial catechol production is a constant battle against toxicity. Biomass could not be measured in organic/aqueous two-phase systems with direct phase contact; consequently, specific production rates could not be determined. Comparison of single-phase aqueous production with the membrane process (Chapter 5) is possible, since here we have a two-phase system with known biomass concentrations.

The maximum specific production rate in the membrane process equals 17 μmol min^{-1} g CDW^{-1} (calculated from Figure 1, Chapter 5), while it was 18 μmol min^{-1} g CDW^{-1} in the single-phase process (calculated from Figure 1, Chapter 4). For these calculations, total product quantities were used. Since growth on LB medium was more pronounced than on minimal media, these maximum specific production rates are generally lower than those in Chapter 2 (minimal media).

In the bioreactor, productivity was somewhat lower than in the small-scale experiments (on a 10-ml scale with LB medium, we found 24 μmol min^{-1} g CDW^{-1}; Chapter 3). However, from the above data it is clear that maximum specific production in a single-phase process is comparable to that in a membrane process with octanol, when total product accumulation is used in the calculations. At maximum productivity, we conclude that toxicity did not differ substantially in these processes.

Volumetric production rates

In this discussion chapter, production rates are calculated differently as compared to the preceeding chapters, to couple volumetric productivity to the culture medium, instead of to the whole liquid volume. In aqueous LB cultures at pH 7, the maximum volumetric production rate was in the order of 0.23 g l^{-1} h^{-1} (calculated from Figure 1, Chapter 4). In LB/octanol two-phase systems with 40% of octanol, this value was approximately 0.10 g l^{-1} h^{-1} (total product divided by total liquid volume) or - on an octanol basis - 0.23 g l^{-1} h^{-1} (product in octanol divided by octanol volume) (calculated from Figure 3, Chapter 4). It is imperative to choose the desired quantity and calculate the correct values.

In two-phase systems, the product partitions over two liquid phases, but it is produced exclusively in the aqueous medium. To obtain an indication of the activity of the biomass, the increase in total amount of 3-methylcatechol in the system was divided by the aqueous volume and the time interval. In this way, aqueous volumetric production rates by the culture were determined (Table 1). The biomass in the two-phase system maximally produced 0.17 g l^{-1} h^{-1} (total product divided by the aqueous volume), which is lower than Q_p in a one-phase system. However, in direct-contact two-phase systems, this $Q_{p,max}$ lasted for 24 hours (Chapter 4), while in one-phase systems the maximum value of 0.23 g l^{-1} h^{-1} was only maintained for one hour (Figure 1, Chapter 4). We do not know how much biomass was present in the two-phase system – it is possible that specific production rates in systems with and without octanol were similar. The maximum volumetric production rate in the membrane process was 0.19 g l^{-1} h^{-1} (total product divided by aqueous volume).

In a single-phase system, the maximum volumetric production rate was thus higher than in a biphasic system. If we compare overall volumetric production rates (over the whole process time), the two-phase process is comparable to the one-phase system (Table 1). Then again, two-phase systems with octanol have advantages for downstream processing (Chapters 4, 5). Production rates may still be improved in the two-phase systems. The lag time has a profound impact on overall volumetric production rates and remains a concern in two-phase processes with direct phase contact (Chapter 4).

Table 1 Volumetric 3-methylcatechol production rates (amount of product divided by aqueous volume and process time) in one- and two-phase systems, to compare the activity of the cultures under different process conditions. The time during which these values were valid is also given.

Process	Maximum volumetric production rate		Overall volumetric production rate	
	$Q_{p,max}$ (g l^{-1} h^{-1})‡	process time	$Q_{p,ov}$ (g l^{-1} h^{-1})‡	process time
One phase (Ch. 4)	0.23	1 h	0.10	12 h
Two phases (Ch. 4)	0.17	24 h	0.09	53 h
Two phases plus membrane (Ch. 5)	0.19	1 h	0.12	12 h

‡ total amount of product, divided by the aqueous (culture) volume and process time

POTENTIAL PROCESSES: DEALING WITH PRODUCT TOXICITY

Apart from the process configurations studied in this research project, other methods might also be considered to achieve the aforementioned goal of an optimal bioprocess for catechol production. To broaden our scope, we will discuss some alternative catechol production processes (Table 2). This overview includes:

- aqueous production
- organic/aqueous two-phase system
- *in situ* product adsorption
- biofilm membrane reactor
- aqueous two-phase system
- continuous-flow stirred-tank reactor (CSTR)
- immobilised cells
- liposome-like structures/membrane vesicles
- cell-free extract/free enzymes
- extreme process conditions
- two-steps production

Aqueous process

Bioproduction of 3-methylcatechol in aqueous media was studied by Robinson *et al.* (1992) and in this thesis. In these batch, small-scale processes, growth of the cells stopped at 5.5 mM of 3-methylcatechol, yet cells remained active and product concentrations as high as 14 mM were obtained (Chapter 2). Production rates and end product concentrations depended on the pH, the growth medium and the concentration of the substrate toluene (Chapter 3). However, the real bottleneck in these aqueous processes (Figure 1A) was the product toxicity towards the biocatalyst, as discussed in the Introduction (Chapter 1).

Organic/aqueous two-phase system

Pseudomonas putida MC2 was tolerant to the presence of a second organic phase. 1-Octanol was chosen to provide the substrate and, more importantly, to extract the product in an organic/aqueous two-phase catechol production process (Figure 1B). This approach was feasible and resulted in improved product concentrations (Chapter 4).

It was tested if a membrane that separates the organic and the aqueous phase (Figure 1C) would prevent to a very large extent phase toxicity of octanol (Chapter 5). Although overall final product concentrations were lower than without a membrane, the volumetric production rate was higher. Furthermore, the octanol phase was - and remained - separate from the culture medium, which was an important advantage for downstream processing. This same set up can also be tested with a different organic solvent, that would dissolve the product even better. Toxicity of the solvent is not as prominent an issue as in the process without a membrane.

In situ product adsorption

Several research groups have studied catechol production in aqueous medium with *in situ* product removal. Robinson *et al.* (1992) tested a variety of adsorptive agents and applied granular activated charcoal to remove 3-methylcatechol from the culture medium (Figure 1B). They found a 3- to 4-fold increase in product yield as compared to a system without adsorption. However, the micro-organisms (*Pseudomonas putida* 2313) and the growth medium constituent glucose were also adsorbed from the medium. The product was easily recovered from the charcoal, using ethyl acetate as eluents, at a recovery exceeding 90%.

Escherichia coli JM101 (pHBP461) was utilised in the production of 3-phenylcatechol by Held *et al.* (1998; 1999). Their bioreactor contents were recycled over a fluidised bed, containing the solid adsorbent Amberlite^{TM} XAD-4, a hydrophobic resin. Various 3-substituted catechols were produced and adsorbed in this set up (Held *et al.*, 1998). Inactivation of the biocatalyst was avoided and a 97% conversion to the desired catechol was obtained (Held *et al.*, 1999).

An activated carbon was used by Woodley and co-workers (Lilly and Woodley, 1996; Lynch *et al.*, 1997) in the production of 3-fluorocatechol from fluorobenzene by *Pseudomonas putida* ML2. Norit pK13 was chosen, because adsorption of product to this sorbent was not reduced by the presence of the substrate fluorobenzene and it was available at particle sizes of 1-3 mm, which allowed a bacterial suspension to pass rapidly without a significant pressure drop. The product was recovered from the column using butyl acetate and the total amount of 3-fluorocatechol produced was about seventy times larger than the minimum toxic level.

These examples show the merit of solid-phase adsorption in the microbial production of toxic catechols. Further downstream processing of the adsorbed product still needs attention.

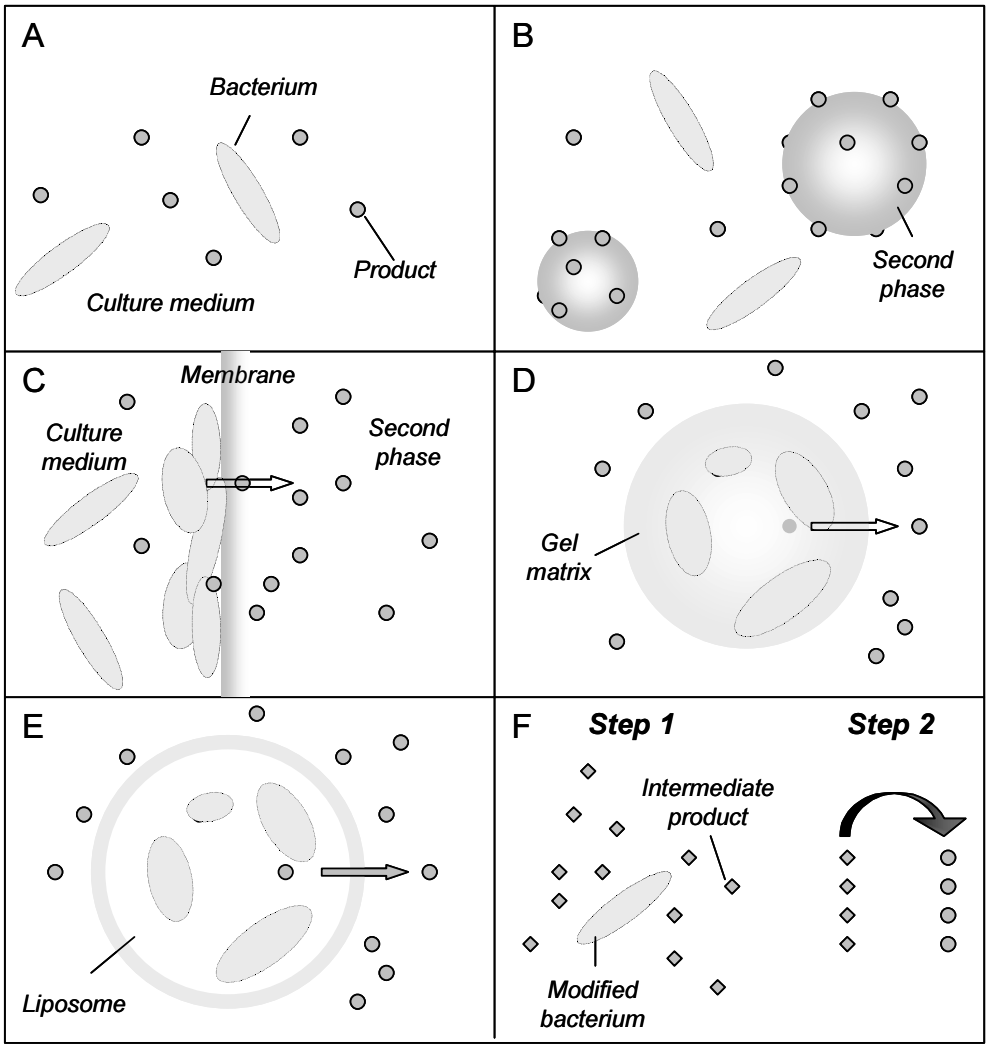

Figure 1 Potential processes for the bioproduction of catechols. A: aqueous production, B: two-phase production, C: membrane bioreactor, D: immobilised cells, E: liposomes/membrane vesicles, F: production in two separate steps.

Biofilm membrane reactor

A membrane configuration in a set up that differs from the set up as described in Chapter 5 might also be used. Use of a biofilm reactor, where the biomass forms a film on the membrane (Reij *et al.*, 1995) might be an alternative in the bioproduction of catechols. The advantages of this method (Figure 1C) can be found in the large interface between the culture medium and the second phase, and in downstream processing, since the two phases are and remain separate in this application. Octanol (or even a more toxic solvent) can be utilised as second phase. This approach is not very different from the approach in Chapter 5. Clogging of the fibres by the growing population has to be avoided.

Aqueous two-phase system

Use of an aqueous two-phase system (Walter *et al.*, 1985) has been considered in our project (Figure 1B). The exploration of an appropriate two-phase system (with respect to biocatalyst activity, to partitioning of substrate and product, to downstream processing, and to stability of the catechol) seemed, however, quite complex and we decided to focus on organic/aqueous two-phase systems with our solvent-tolerant biocatalyst. However, this aqueous two-phase approach may be an option in the bioproduction of some catechols, depending on the product hydrophilicity.

Continuous-flow stirred-tank reactor (CSTR)

A continuous, constant feed flow, with simultaneous extraction of medium from the fermentor – keeping the aqueous volume constant – can be applied in different ways. The rationale of using this approach is to keep the aqueous product concentration near the biocatalyst low, while at the same time the product is continuously delivered to a downstream processing unit. In an aqueous one-phase process, a CSTR can be implemented straightforwardly and toxicity problems for the cells can be reduced (Figure 1A). Also in the two-

phase process with a membrane barrier (Chapter 5), this option may be considered (Figure 1C). However, the aqueous phase gets diluted in this approach, and this is not desirable for efficiently obtaining the product. Therefore, we do not consider this option to be a good alternative process.

Continuous replenishment of octanol might improve the process if very high product concentrations are obtained.

Immobilised cells

The biocatalyst can be immobilised (Figure 1D), to protect cells from solvent toxicity in organic/aqueous two-phase systems (León *et al.*, 1998). Mass transfer resistances nevertheless are enhanced as compared to submerged cultures. Although some synthetic polymers can provide stable matrices, problems of gel instability in the presence of organic solvents (which in our case not only involves octanol, but also toluene), partition and mass transfer limitations, and insufficient mechanical stability have been reported, as well as only slight protection against solvent toxicity (León *et al.*, 1998). Furthermore, product accumulation inside the immobilisation matrix will limit the activity of the cells. Another difficulty in our process is the need for growing cells, which implies that not only the substrate for the reaction, but also growth substrate has to diffuse at a sufficiently high rate. Growth of the cells might eventually lead to eruption of bacterial colonies in the culture medium (Hüsken *et al.*, 1996), that would impede downstream processing.

Immobilisation is useful in some cases, but in our bioprocess this option seems not relevant, in particular in view of undesirable local product accumulation.

Liposome-like structures/membrane vesicles

Whole-cell micro-encapsulation (Figure 1E) is based upon the occlusion of cells inside hollow micro-spheres of a (polymeric) membrane (León *et al.*, 1998). The hollow space can be filled with an aqueous solution or a hydro gel. This method can enhance mass transfer rates and the stability of the support.

The continuous phase surrounding the microspheres should extract the product, to prevent product inhibition for the cells and to facilitate downstream processing. Growth of the biocatalyst inside the microsphere, necessary for high activity of *P. putida* MC2, requires the presence and transport of growth substrate, apart from the reaction substrate (toluene) in membrane vesicles.

Cell-free extract/free enzymes

One of the reasons we chose to work with whole cells, is the instability of the dioxygenase enzyme complex, needed in the first step of toluene degradation (Collins *et al.*, 1995). To work with free enzymes would therefore be extremely complex and not possible for a prolonged period of time (Table 2). *P. putida* MC2 is a stable biocatalyst. Whole cells provide the perfect physiological conditions for the pertinent enzymes, and ensure co-factor regeneration (Chapter 1, Figure 3).

Extreme process conditions

Changing temperature or pressure in a production process often has a large impact on the outcome of the process. In biocatalysis, the process conditions can be altered to a certain limit only. Maintaining a physiologically tolerable temperature is important to keep bacterial cells alive and active. Since we focus on biological catechol production, and try to promote “green chemistry” (Chapter 1, Table 1, line 6), the available room for manoeuvre is small and ambient conditions are preferred.

Nevertheless, some bacteria are capable of surviving extreme conditions, while maintaining activity. For example, the extremophilic bacterium *Pyrococcus furiosus* thrives at very high temperatures, while other extremophiles are active at extreme pH values. These kinds of organisms can thus be used at atypical reaction conditions (Table 2). In a way, *P. putida* MC2 is also an extremophile, because it withstands the presence of a second phase of octanol.

Two steps production

If a biocatalyst would be utilised that contains the *todCBA* genes (encoding for the toluene dioxygenase; see Chapter 1, Figure 4), but not the *todD* gene (encoding for the dehydrogenase), the intermediary toluene *cis*-glycol would be accumulated (Figure 1F, step 1). This intermediary in the catechol production process is water-soluble and not toxic to *P. putida* cells (Collins *et al.*, 1995; Lynch *et al.*, 1997; Robinson *et al.*, 1992) and it is therefore reasonable to assume that it can be accumulated to higher concentrations than catechols. Whole cells are needed, with for instance ethanol as a co-substrate (Collins *et al.*, 1995), for the supply of stoichiometric amounts of co-factor NADH (Chapter 1, Figure 4).

To obtain the desired catechol, the dehydrogenation step has to be carried out subsequently (Figure 1F, step 2). This might be done biologically, by means of a bacterium that expresses the dehydrogenase, and again regeneration of intracellular NADH to NAD^+ must be implemented. However, the biological follow-up reaction after the production of the non-toxic intermediary toluene *cis*-glycol (in a reaction environment apart from the first step) most likely poses toxicity problems to the second biocatalyst that are similar to the effects described for our *P. putida* strain MC2. The second step can also be carried out by a chemical reaction (this step is chemically uncomplicated as compared to the first step in region-selective 3-substituted catechol production), as proposed by Robinson *et al.* (1992). If the use of an organic solvent must be prevented, aqueous production of toluene *cis*-glycol by a *P. putida* strain, deprived of functional *todD* genes, would be possible, followed by a separate second step to obtain the catechol.

In Table 2, strengths and weaknesses of the above bioproduction processes are listed.

Table 2 Comparison of weaknesses and strengths of different (toxic) bioprocesses

Process	Weakness	Strength
Aqueous production	▪ accumulation of toxic product near biocatalyst ▪ product instability	▪ fast process
Organic/aqueous two-phase systems	▪ long lag time ▪ toxicity aspects of the organic phase	▪ high volumetric production rate ▪ high product concentration in separate phase ▪ product stability ▪ facilitated DSP
In situ product adsorption	▪ extra elution step needed	▪ increased product yield
Biofilm membrane reactor	▪ possible clogging of membrane fibres	▪ high volumetric production rate ▪ high product concentration in separate phase ▪ product stability ▪ facilitated DSP
Aqueous two-phase system	▪ product instability ▪ not appropriate in production of hydrophobic catechols	▪ stable and moderate conditions for cells
Continuous-flow stirred-tank reactor (CSTR)	▪ dilution of aqueous phase – decreased product concentrations	▪ decreased product-toxicity problems
Immobilised cells	▪ product accumulation near biocatalyst ▪ enhanced mass transfer resistances ▪ possible gel instability, partitioning and mass transfer limitations ▪ contamination of continuous phase by growing biomass, impeding DSP	▪ diminished solvent toxicity
Liposome-like structures/ membrane vesicles	▪ limited growth of cells	▪ decreased product toxicity ▪ enhanced mass transfer rates possible
Cell-free extract/free enzymes	▪ instable enzymes ▪ co-factor regeneration might need more attention	▪ a fast process and less inhibition might be obtained
Extreme process conditions	▪ environmental impact	▪ atypical reaction conditions possible
Two-steps production	▪ more complex process ▪ co-factor regeneration must be provided	▪ higher product concentrations realisable ▪ ambient conditions; no need for organic phase

PRODUCTION OF OTHER 3-SUBSTITUTED CATECHOLS

We focussed on the production of 3-methylcatechol as a model reaction. The toluene-degrading enzymes are not very specific and can convert more substrates than toluene. Horlings (1999) made an effort to produce different catechols in aqueous and in organic/aqueous systems. Production of 3-ethylcatechol and 3-fluorocatechol by *P. putida* MC2 was accomplished (the latter more pronounced), while production of 3-nitrocatechol – a very interesting compound for the fine-chemical industry (Chapter 1) – was limited.

INDUSTRIAL RELEVANCE

As explained in the Introduction, catechols play an important role in the fine-chemical and flavour industry, as well as in photography, dyeing fur, rubber and plastic production (Chapter 1). Chemical production of 3-substituted, relevant catechols is hindered and results in mixtures of 3- and 4-substituted catechols, whereas some micro-organisms are able to specifically produce the desired catechol (Chapter 1). Therefore, industrial microbial production – in one of the methods described above – can be a good alternative for the production of these relevant molecules and could result in a pertinent production process.

CONCLUDING REMARKS

In this thesis, we showed that biological production of 3-substituted catechols is possible in a number of process configurations. Depending on the crucial parameter (substrate, reactor, biomass, or downstream processing costs), the process can be optimised and the appropriate set up can be chosen.

Microbial catechol production is a constant battle against toxicity. However, as we explained in this thesis, the effective combination of microbial and process-engineering principles offers bioprocesses at high productivities.

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Summary

Catechols play an important role in the fine-chemical and flavour industry, as well as in photography, dyeing fur, rubber and plastic production. Many of these compounds cannot easily be synthesised chemically, but some micro-organisms are capable of producing catechols from an aromatic compound in only two steps. In this thesis, microbial 3-methylcatechol production from toluene is considered a model system for simulating the production of 3-substituted catechols in general (Chapter 1).

Microbiological and engineering aspects of 3-methylcatechol production were studied. *Pseudomonas putida* MC2 was a newly constructed strain that was capable of producing 3-methylcatechol from toluene in two steps, in the presence of inducer salicylate (Chapter 2). This trait was stably expressed and no antibiotics were required to maintain this stability. Growing cells produced the desired product at very high specific production rates (Chapters 2, 3).

In aqueous medium at one-litre scale, 10 mM of 3-methylcatechol accumulated in twelve hours (Chapter 4). Toxicity of the product, however, prevented further production. In addition, the stability of the product in aqueous medium at physiological pH was poor and downstream processing of the culture medium containing the product was complicated. Therefore, other process set ups were studied as well.

P. putida MC2 was tolerant to some organic solvents that are toxic to micro-organisms in general. Octanol was a solvent in which substrate toluene and product 3-methylcatechol both dissolved very well, while the biocatalyst survived and was active in its presence. Extraction of the product to an octanol phase was advantageous for downstream processing in comparison to monophasic aqueous processes. Biphasic 3-methylcatechol production resulted in improved volumetric production rates and higher product concentrations. Up to 65 mM of product accumulated in octanol, and a maximum overall product concentration (over octanol and aqueous volume

together) of 25 mM was obtained at a one-litre scale, depending on the volume ratio of both liquid phases (Chapter 4).

The two-phase process, with octanol in direct contact with the culture medium, started slowly. A lag time of 24 hours (depending on the phase ratio) before production started was not unusual. Molecular toxicity of dissolved octanol in the culture medium was negligible. We thus assumed that the direct contact with octanol was very inhibitory to the bacteria (phase toxicity). To avoid toxic phase effects of the second phase, a membrane was introduced between both phases (Chapter 5). In this process configuration, a lag was virtually absent and the total process was faster than without a membrane. Maximum volumetric production rates rose 40 %, albeit that the product concentration in octanol was not as high as in the process with direct phase contact. Mass transfer in the membrane process thus still needs attention.

Preliminary experiments on the downstream processing of 3-methylcatechol from octanol were carried out (Chapter 5). The disodium salt of the product was extracted into a sodium hydroxide solution and was separated from the octanol phase that still contained the toluene and that could be recycled to the process. The salt solution was acidified to reprotonate the product, which was now stably captured at high concentration in an aqueous acidic phase (Chapter 5). A new organic solvent that dissolves the product well and that would be more volatile than octanol, for instance dichloromethane, can be used to obtain the purified product after phase separation and solvent evaporation.

To attain a rational design and to be able to further optimise this process, a process model was developed, both for one- and two-phase processes. The importance of different process parameters on bacterial growth, on product accumulation in both phases and on process time follow from the model (Chapter 6). It seemed the process would be faster (14 – 27 % for one- and two-phase process, respectively) if more growth substrate would be available, although this hardly affected the final product concentrations.

The general discussion (Chapter 7) compares production rates and gives an overview of alternative 3-methylcatechol production processes. It furthermore discusses the industrial relevance and presents concluding remarks.

Samenvatting

Catecholen zijn belangrijke bouwstenen in de fijnchemie, geur- en smaakstoffenindustrie, fotografie, rubber- en plasticproductie. Vaak kunnen ze niet eenvoudig chemisch gesynthetiseerd worden, maar er zijn micro-organismen die catecholen kunnen produceren in slechts twee stappen, uitgaande van een aromatische verbinding. Microbiële productie van 3-methylcatechol staat in dit proefschrift model voor de productie van 3-gesubstitueerde catecholen in het algemeen (hoofdstuk 1).

Microbiologische en procestechnologische aspecten van 3-methylcatecholproductie zijn bestudeerd. *Pseudomonas putida* MC2 was een nieuwe bacterie die in twee stappen 3-methylcatechol kon produceren uit tolueen, in aanwezigheid van de inductor salicylzuur (hoofdstuk 2). Deze stam was erg stabiel en er was geen antibioticum nodig om deze eigenschap in een groeiende cultuur te handhaven. Cellen produceerden het gewenste product bij hoge specifieke productiesnelheden (hoofdstukken 2, 3).

In waterig medium hoopte in twaalf uur op één literschaal 10 mM 3-methylcatechol op. Toxiciteit van het product voorkwam verdere productie. Daarnaast was de stabiliteit van het product bij fysiologische pH niet hoog en was de opwerking ingewikkeld. Daarom zijn er ook alternatieve processen bestudeerd.

P. putida MC2 was tolerant voor enkele – doorgaans toxische – organische oplosmiddelen, zoals octanol. Zowel tolueen als 3-methylcatechol lossen veel beter op in octanol dan in waterig medium. Om toxische effecten tegen te gaan werd gekozen voor een twee fasenproces, waarbij octanol in direct contact was met het waterige medium waarin zich de biokatalysator bevond. Voor de opwerking was het een voordeel dat het product in een octanolfase zat, in vergelijking met waterige processen. Productie van 3-methylcatechol in aanwezigheid van een octanolfase resulteerde in verbeterde volumetrische productiesnelheden en hogere productconcentraties. Tot 65 mM product hoopte op in octanol en een maximale totale productconcentratie (gemeten

over octanol en waterig volume samen) van 25 mM werd verkregen op één literschaal, afhankelijk van de volumeverhouding (hoofdstuk 4).

Een nadeel van dit twee fasenproces was dat de productie traag op gang kwam. Een *lag* tijd van 24 uur voordat de productie startte was niet ongewoon. Moleculaire toxiciteit van opgelost octanol in het waterige medium was verwaarloosbaar. Daarom werd aangenomen dat het directe contact met octanol erg remmend werkte voor de bacterie (fasetoxiciteit). Om toxische fase-effecten van de tweede fase te voorkomen werd een membraan tussen beide fasen geïntroduceerd (hoofdstuk 5). In deze procesconfiguratie werd geen *lag* tijd gevonden. De maximale volumetrische productiesnelheden namen met 40 % toe, hoewel de productconcentratie in octanol minder hoog was. Stofoverdracht in het membraanproces was dus niet optimaal.

Enkele oriënterende experimenten rond de opwerking van 3-methylcatechol uit octanol werden uitgevoerd (hoofdstuk 5). Het dinatriumzout van het product werd geëxtraheerd in een natronloogoplossing en werd gescheiden van de octanolfase, die nog steeds tolueen bevatte en kon worden hergebruikt in het proces. De zoutoplossing werd aangezuurd om het product weer te protoneren. Het product bleef daarbij stabiel en het werd in hoge concentratie gevangen in deze zure oplossing (hoofdstuk 5). Een ander organisch oplosmiddel waarin het product goed oplost en dat vluchtiger is dan octanol, bijvoorbeeld dichloormethaan, kan gebruikt worden om het zuivere product in handen te krijgen, na fasescheiding en verdampen van dat oplosmiddel.

Om een rationeel procesontwerp te krijgen en het proces verder te kunnen optimaliseren werd een model ontwikkeld, zowel voor één als voor twee fasenprocessen. Het effect van verschillende procesparameters op groei, productophoping in beide fasen en procestijd volgde uit het model (hoofdstuk 6). Volgens het model zou het proces sneller zijn (14 – 27 % voor één en twee fasen, respectievelijk) als meer groeisubstraat werd toegevoegd, hoewel dit nauwelijks effect op de uiteindelijke productconcentraties had.

In de algemene discussie (hoofdstuk 7) zijn productiesnelheden vergeleken en is een overzicht opgenomen van alternatieve biologische 3-methylcatechol-productieprocessen. Tevens wordt de industriële relevantie besproken en worden concluderende slotopmerkingen gemaakt.

Nawoord

Het boekje is af! Alsof je na een lange, zware wandeling in de nabije verte de langverwachte berghut ziet liggen. Met de belofte van Schiwasser, koud bier, Milka en goulashsoep lijken die laatste hoogtemeters minder steil en voelt de rugzak minder zwaar. Omdat deze tocht door velen werd ondersteund lijkt een “dank je wel” me wel op zijn plaats.

Allereerst degenen die mede de route uitstippelden en voorkwamen dat ik in de mist verdwaalde. Bij dit project van Industriële Microbiologie (IM) en Proceskunde (PK) waren twee promotoren en twee co-promotoren betrokken. Jan, je nam het me kwalijk dat ik “nog geen postzegel over had” voor een sollicitatie naar dit project en ik zei ook niet meteen enthousiast “ja” toen je me vroeg AIO te worden. Toch is het een goede tijd geworden en heb ik geen spijt gehad van deze keuze. Ik waardeer het zeer dat je ook na de ingrijpende reorganisatie en je vertrek van deze universiteit promotor bent gebleven. Hans, het was me een genoegen dat ook jij promotor was. Je wist me als student al zeer te boeien met het vakgebied van de bioprocestechnologie en ik kreeg als AIO de mogelijkheid me hier verder in te verdiepen.

Rik, al ben ik niet zo goed in gezegdes als jij, meestal spreek je duidelijke taal. Nuchter en “to the point”. Dat werkt heel verhelderend – vaak ook op je lachspieren - en is een prettige manier van communiceren. Daarnaast spreek je de taal van de beide secties en kwam ik altijd goedgemutst uit onze besprekingen. Jan, mijn gebrekkige kennis van genetica en jouw nieuwsgierigheid naar proceskunde leidden tot vele nuttige besprekingen, met name rond hoofdstuk 2 en 3. Op het lab werd veel gelachen, mede door je vrolijke gezang (met Harald) – altijd nagesynchroniseerd naar het Nederlands.

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Bij IM (met negen AIO's en een paar PC's op een kamer) was de sfeer fantastisch. Tijdens de excursie naar New Jersey en IJsland, de kerstlunches, labuitjes en Veluweloop, maar ook gewoon op het lab was het altijd dolle pret. Het viel ongelooflijk rauw op ons dak toen we hoorden dat bij deze universiteit geen toekomst meer was voor IM. Lof voor iedereen die in die tijd IM draaiende hield en de sfeer wist vast te houden. De "reünies" zijn heel gezellig!

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Leonie

Curriculum vitae

Leonie Engeline Hüsken werd geboren op 14 mei 1973 te Enschede. In 1991 behaalde zij haar VWO-diploma aan het Thijcollege te Oldenzaal. In datzelfde jaar startte zij haar studie Bioprocestechnologie aan de Landbouwuniversiteit in Wageningen. Na afstudeervakken bij de secties Proceskunde en Industriële Microbiologie aan de LUW en een stage Proceskunde bij Unilever Research in Vlaardingen, rondde zij haar studie af in september 1996.

Vervolgens werd zij in januari 1997 AIO bij de secties Industriële Microbiologie en Proceskunde aan de LUW. Het project waaraan zij werkte integreerde de disciplines van beide secties. Het onderzoek dat zij hier uitvoerde heeft geleid tot dit proefschrift.

Vanaf december 2001 is zij in dienst bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) te Den Haag, voor het gebied Exacte Wetenschappen.

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