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## Optimization of the hydroxylation of 2-cyclopentylbenzoxazole with *Cunninghamella blakesleeana* DSMZ 1906

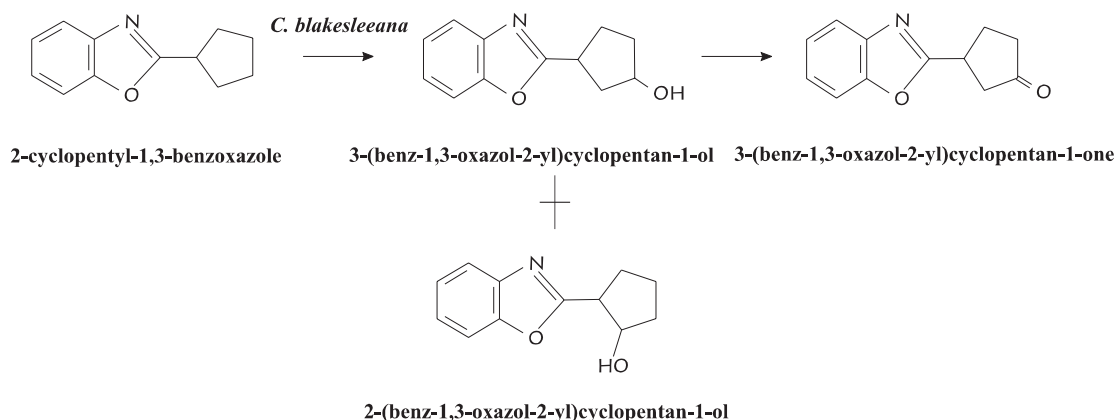
**Abstract** Biohydroxylation of 2-cyclopentyl-1,3-benzoxazole with the filamentous fungus *Cunninghamella blakesleeana* DSMZ 1906 was studied in a 15-l stirred tank reactor. The aim of the work was to avoid substrate limitation through sub-optimal mixing by formation of pellets with a uniform pellet size distribution of 250–500  $\mu\text{m}$ , obtained at an inoculum concentration of  $10^7$  spores  $\text{ml}^{-1}$  and an agitation rate of 390 rpm. Due to the high toxicity of the educt, 2-cyclopentyl-1,3-benzoxazole, on the fungus, the medium composition, the time of educt addition, and the educt starting concentration were optimized to reach high educt tolerance and hydroxylation activity. A good maintenance of biotransformation capacity was obtained without excessive loss of activity of the biocatalyst by addition of 30 mg 2-cyclopentyl-1,3-benzoxazole/g biomass (cell dry mass) during the stationary phase in a medium which was optimized in batch fermentations with experimental designs. An increase in product yield and quality (enantiomeric excess) was achieved by developing feeding strategies combining the educt and medium components. The resulting fermentation broth contained  $450 \text{ mg l}^{-1}$  of the product (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol with an enantiomeric excess of 95%, which represents a 48% increase over former reported results.

### Introduction

Biohydroxylation represents a powerful method for the regioselective and stereoselective introduction of hydroxyl groups into organic compounds (Holland 1992). As such, it can be viewed as an intermediate step for any other subsequent functionalization. In this kind of biocatalytic process, filamentous fungi, especially the Zygomycete *Cunninghamella blakesleeana* DSMZ 1906, have been widely used (Kreiner et al. 1996; Zhang et al. 1996; Pothuluri et al. 1997).

A method often applied for the synthesis of pharmaceuticals is the microbial oxidation of 2-cyclopentyl-1,3-benzoxazole (CPB) into 3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol (Fig. 1), a model reaction for cycloalkanecarboxylic acids. Its main advantages are a good educt availability, known reaction steps and established analytics of the main products (deRaadt et al. 1996a, 1996b, 1996c). Kreiner et al. (1996) have performed the initial screening of the regiospecific and stereospecific hydroxylation activity of the fungus. Subsequently, the main product formation, (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol, by *C. blakesleeana* was optimized to gain high enantiomeric excess (e.e.). It was then important to control the special morphological features of the fungus in order to attain reproducible high quantities of the desired diastereomer. Furthermore, the environmental conditions need to be optimized in order to resolve the problem of educt and metabolite intolerance and to obtain a biocatalyst with high activity and stability.

*C. blakesleeana* grows either in filamentous or in pellet form. A uniform pellet size is preferred, since it provides reproducible transformation conditions in the stirred batch reactor. The optimal size of the pellets is less than 400  $\mu\text{m}$  diameter, so that diffusion limitations are avoided within the pellets (Kobayashi et al. 1973; Wittler et al. 1986). However, the pellet size may be influenced by a number of factors. Among these are the inoculum size, the growth medium, and environmental



**Fig. 1** Hydroxylation of 2-cyclopentyl-1,3-benzoxazole (CPB) by *Cunninghamella blakesleeana*. Structural formulae of products: 3-(benz-1,3-oxazol-2-yl)-cyclopentan-1-ol (product), 2-(benz-1,3-oxazol-2-yl)-cyclopentan-1-ol (by-product 1) and 3-(benz-1,3-oxazol-2-yl)-cyclopentan-1-one (by-product 2)

conditions, i.e. rates of agitation and aeration (Whitaker and Long 1973; Braun and Vecht-Lifshitz 1991; Znidarsic et al. 1998).

The aim of the present work is the development of a new feeding strategy, which includes a combination of nutrient feed and toxic educt addition. This should provide a stable and highly active biocatalyst enabling a high final product concentration with good yield and high e.e.. A range of starting concentration levels of CPB and different timings of CPB addition are therefore investigated.

## Materials and methods

### Strain and culture conditions

*C. blakesleeana* DSMZ 1906 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. Stock cultures were grown at room temperature in the dark on potato/dextrose (Biolife) agar slants and subcultured every four weeks. Each shake flask or fermentation experiment was inoculated under sterile conditions with a spore suspension (final concentration approx.  $10^7$  spores  $l^{-1}$ ) prepared from agar-slant cultures grown for three weeks.

### Medium composition and fermentation procedure

#### Medium

The fermentation medium, which was developed from shake flask experiments, consisted of glucose 10 g  $l^{-1}$ , peptone M66 5 g  $l^{-1}$ , malt extract 0.3 g  $l^{-1}$ ,  $NH_4Cl$  2.9 g  $l^{-1}$ ,  $Na_2HPO_4$  2.4 g  $l^{-1}$ ,  $KH_2PO_4$  2.4 g  $l^{-1}$ ,  $MgSO_4 \cdot 7H_2O$  1.3 g  $l^{-1}$ ,  $CaCl_2 \cdot 2H_2O$  0.1 g  $l^{-1}$ , iron(III) citrate hydrate 0.04 g  $l^{-1}$ ,  $ZnSO_4 \cdot 7H_2O$  0.02 g  $l^{-1}$ ,  $CuSO_4 \cdot 5H_2O$  0.03 g  $l^{-1}$ . All chemicals were obtained from Merck (p.a. quality). Media were prepared using distilled water and sterilized in an autoclave.

Experimental designs were performed in two parallel runs in a rotary shaker (120 rpm, 30 °C) in 300-ml baffled shake flasks containing 100 ml medium. The factorial screening design ( $2^4$ ) employed two levels with one center point. Data analysis was performed with Statgraphics 3.0 *plus*. Tested complex nitrogen

sources in the first design were yeast extract, malt extract, peptone M66 and meat extract at 0, 2.5 g  $l^{-1}$  and 5 g  $l^{-1}$ . The inorganic component calcium (0, 0.125 g  $l^{-1}$  and 0.25 g  $l^{-1}$  concentration levels) and the trace elements manganese, copper and molybdenum (0, 0.02 g  $l^{-1}$  and 0.04 g  $l^{-1}$ ) were tested in the second experimental design.

Further investigation of the medium components peptone M66, malt extract,  $NH_4Cl$ ,  $MgSO_4 \cdot 7H_2O$ , iron(III) citrate hydrate and  $ZnSO_4 \cdot 7H_2O$  was done with the "genetic algorithm" (12 individuals, four generations, selection method "keep the best and roulette on the rest"), using Galop software from FZ Jülich (Beste et al. 1992; Karutz et al. 1992).

### Bioreactor and fermentation procedure

Experiments were performed in a 15-l stirred tank bioreactor (Biostat E, Braun, Melsungen, Germany) with 10 l working volume. Temperature was monitored and held at  $30 \pm 0.1$  °C. pH was measured with a glass electrode (Ingold, Urdorf, Switzerland) and controlled at  $6.0 \pm 0.1$  through addition of either 15% (mass/vol.) NaOH or 15% (mass/vol.)  $H_3PO_4$ . The aeration rate was kept at 1 vvm during the growth phase and at 0.5 vvm during the production phase by adjusting the gas-flow rate. The agitation rate was kept constant during the fermentation. The influence of the agitation rate was tested at 160, 390, 550 and 730 rpm (0.61, 1.38, 2.10 and 2.79  $ms^{-1}$  stirrer tip speed, respectively). CPB was added aseptically giving a starting concentration of 150, 350 or 1000 mg  $l^{-1}$  in the fermentation broth. CPB was provided by the Institute of Organic Chemistry, TU Graz (de Raadt et al. 1996b).

### Analytical methods

Biomass was determined as cell dry mass (CDW) by vacuum filtration using a cellulose acetate filter (0.45  $\mu m$  pore size) and drying with a moisture analyzer (SATORIUS MA30) at 105 °C until constant mass.

The pellet size distribution was estimated with Retsch sieves. After sieving the pellet suspension for 20 min and simultaneously gently washing with water, the masses of the different fractions were determined as CDW. Morphological observation was done with an Olympus phase-contrast microscope BSH2.

Glucose and organic acids were determined from the supernatant of the fermentation broth (centrifuged at 14800 g, 10 min, 4 °C) by HPLC (Merck IR detector L-7490 and ultraviolet detector L-7400, pump L-7100, column HPX-87H, eluent 0.005 M  $H_2SO_4$ , flow rate 0.6 ml  $min^{-1}$ , 60 °C, ultraviolet detection at 210 nm). Concentrations of CPB and its metabolites were monitored by gas chromatography with injector and detector temperatures of 250 °C and 280 °C, respectively (Hewlett Packard HP 5890 series II, autosampler HP 6890 Series Injector, FI-detector and HP 5 column). The oven temperature was held at 200 °C for 3 min, then heated at a rate of 10 °C  $min^{-1}$  to 250 °C and held for 5 min. Samples were prepared as described by Kreiner et al. (1996).

e.e., which was defined as the excess of the major enantiomer in relation to both enantiomers, was determined by chiral HPLC using the equipment and methods described by deRaadt et al. (1996c).

## Results

### Media optimization

The investigated medium components, which were selected from the literature (Sedlacek et al. 1981; Venkateswerlu et al. 1989; Hartman et al. 1990; Kreiner et al. 1996), were tested in shake flask experiments in two screening designs. An average value of  $4 \pm 2 \text{ g l}^{-1}$  CDW was achieved in the shake flasks containing a combination of different nitrogen sources, in sum  $10 \text{ g l}^{-1}$ . The combination of peptone M66 ( $5 \text{ g l}^{-1}$ ) and malt extract ( $5 \text{ g l}^{-1}$ ) had a positive influence on the biomass concentration after 48 h ( $7.3 \text{ g l}^{-1}$ ). This combination of complex nitrogen sources was used in the second design. None of the tested inorganic media components had a significant influence on the biomass production with an average of the final CDW of  $7.5 \pm 0.8 \text{ g l}^{-1}$  after 48 h.

In subsequent shake flask experiments, which were planned using a genetic algorithm with the improvement of the CPB conversion rate as the target function, the resulting yield of the main product (60%) after 20 h transformation remained constant, although the concentration of malt extract was diminished from  $5 \text{ g l}^{-1}$  to  $0.3 \text{ g l}^{-1}$ .

In these experiments, the obtained pellets were hollow, with a diameter of 0.5–7 mm; the final pH of the broth, which was not controlled, varied over pH 3–7.5.

### Pellet formation and influence of the agitation rate

Three hours after inoculation of the bioreactor, the unstructured spores started swelling and branching in the culture medium. The growth of a densely branched mycelium with a large hyphal diameter was accompanied by NaOH consumption for pH adjustment after 7 h. In contrast to the results with other fungi (Tough and Prosser 1996; Pothuluri et al. 1997), acids such as acetate, pyruvate, maleate, oxalate, citrate, succinate, fumarate or malate were not found by HPLC analysis. The maximum growth rate, depending on the agitation speed (Table 1), was achieved after 15 h fermentation. Pellet formation started at the end of the exponential

growth phase after approximately 18 h. The resulting pellets consisted of a loose network of hyphae.

Different agitation speeds resulted in changes of morphological and kinetic properties (Table 1). At low agitation speed (160 rpm), the fungus showed filamentous growth. Impeller speeds higher than 340 rpm induced pellet formation. The main fraction of pellets in all fermentation experiments had a diameter of 250–500  $\mu\text{m}$ , and the percentage of pellets  $\geq 250 \mu\text{m}$  decreased slightly with increasing agitation speed.

### Biotransformation

Due to the high toxicity of CPB to the fungus, its start concentration and the time point of addition were examined in the optimized fermentation medium at 390 rpm agitation speed. CPB was added to the fermentation broth, giving start concentrations of 150, 350 or  $1000 \text{ mg l}^{-1}$  (30, 60,  $200 \text{ mg g}^{-1}$  CDW, respectively) after 26 h when the fungus was in the stationary phase. The optimal starting concentration was  $350 \text{ mg l}^{-1}$  regarding transformation rate, and  $150 \text{ mg l}^{-1}$  regarding catalyst stability (Table 2). CPB adsorbs immediately after the addition on the biomass, which explains the rapid decrease of substrate concentration in the supernatant. The main product and by-product 1 formation started immediately, and the formation of by-product 2 started with slight delay (30 min) after product formation (Fig. 2). The e.e. of the main product increased rapidly from almost racemic to approximately 80% of its final value (Fig. 5). Further conversion rate was rather slow. The enhancement of the e.e. of the main product was accompanied by an increase in by-product 2 concentration (Fig. 2, Fig. 5).

CPB addition in the transition phase (from exponential growth to the stationary phase, after 19 h) and in the stationary phase (after 26 h) resulted in nearly the same conversion rates and a high final e.e. of the main product. The stability of the biocatalyst against the toxic educt CPB was higher in the stationary phase than in the transition phase (Fig. 3, Table 2).

### Feeding profile

A feeding strategy was developed in order to increase the final concentration of the product and to maintain biocatalyst stability. CPB was added in three pulses starting in the stationary phase (26, 35, 44 h), and each pulse

**Table 1** Influence of the agitation rate  $U$  on characteristic fermentation parameters (maximum growth rate  $\mu'_{\max}$ , death rate  $k'_{\text{deakt}}$ , formation rate  $r_P$  of the product, all calculated after Moser

$U$ [rpm]	$d_{250 \mu\text{m}}$ [%]	$\mu'_{\max}$ [ $\text{h}^{-1}$ ]	$k'_{\text{deakt}}$ [ $\text{h}^{-1}$ ]	$r_P$ [ $\text{mg l}^{-1} \text{ h}^{-1}$ ]	e.e. [%]
160	ND	$0.32 \pm 0.02$	$0.06 \pm 0.02$	$47 \pm 5$	$84 \pm 1$
390	$79 \pm 6$	$0.36 \pm 0.02$	$< 0.02$	$30 \pm 3$	ND
550	$53 \pm 6$	$0.42 \pm 0.02$	$0.02 \pm 0.02$	$45 \pm 4$	$82 \pm 1$
730	$53 \pm 6$	$0.50 \pm 0.02$	$0.14 \pm 0.02$	$42 \pm 4$	$63 \pm 1$

and Steiner (1975); percentage of pellets with a diameter of 250  $\mu\text{m}$  and 500  $\mu\text{m}$   $d_{250 \mu\text{m}}$ , and e.e. of 1*S*,3*S*-diastereomer). ND, not determined

**Table 2** Influence of the starting concentration and time point of adding 2-cyclopentyl-1,3-benzoxazole (CPB) on fermentation parameters (death rate  $k'_{\text{deakt}}$ , formation rate  $r_p$  of the main product and e.e. of the (1*S*,3*S*)-diastereomer). ND, not determined

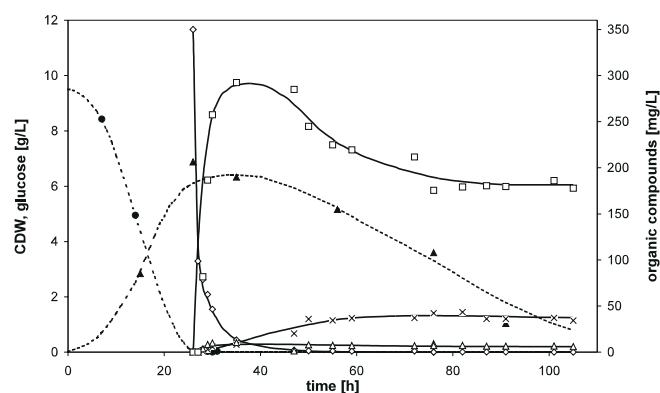
CPB [mg l <sup>-1</sup> ]	$k'_{\text{deakt}}$ [h <sup>-1</sup> ]	$r_p$ [mg l <sup>-1</sup> h <sup>-1</sup> ]	e.e. [%]
150 (19 h)	0.05 ± 0.02	30 ± 3	85
150 (26 h)	0.01 ± 0.02	30 ± 3	82
350 (26 h)	0.05 ± 0.02	110 ± 11	70
1000 (26 h)	0.13 ± 0.02	0.49 ± 0.05	ND

created a final concentration of 100–150 mg l<sup>-1</sup> (30 mg g<sup>-1</sup> CDW). The final product concentration of 250 mg l<sup>-1</sup> in the supernatant was reached without rapid cell lysis (Fig. 3). The e.e. of the product was increased from 71% in the unpulsed process to 82% in the pulsed process and reached its maximum value after 100 fermentation hours (Fig. 5). The biomass concentration remained stable during the three pulses, then the CDW concentration dropped down from 5.2 g l<sup>-1</sup> to 3.0 g l<sup>-1</sup> within 26 h after the last CPB pulse (Fig. 3).

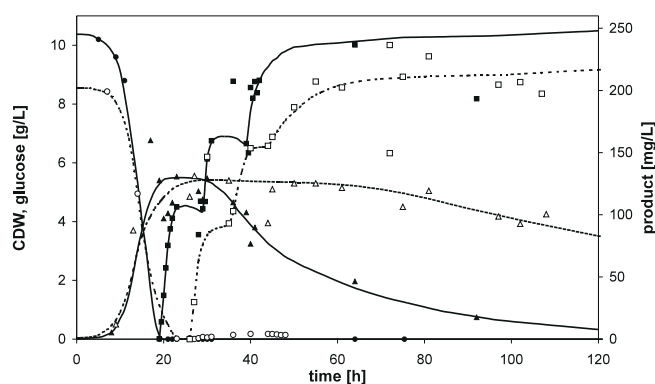
For extended, long-term experiments, a stable biocatalyst concentration with high activity is essential. Therefore, the biomass concentration was kept in the range 5.5–8.5 g l<sup>-1</sup> by regeneration with a glucose feed between the CPB pulses. When the product formation rate rapidly slowed, glucose feeding was started, which was accompanied by increasing oxygen consumption. The feed was stopped when the oxygen demand decreased again. After 30 min recreation time for the biomass, the next CPB pulse was added (Fig. 4). The resulting product concentration of 450 mg l<sup>-1</sup> was achieved after six CPB pulses. The e.e. of by-product 2 decreased with the fermentation time and the e.e. of the main product reached a final value of 95% (Fig. 5).

## Discussion

In shake flask experiments, the medium was optimized but the morphology of the fungus could not be

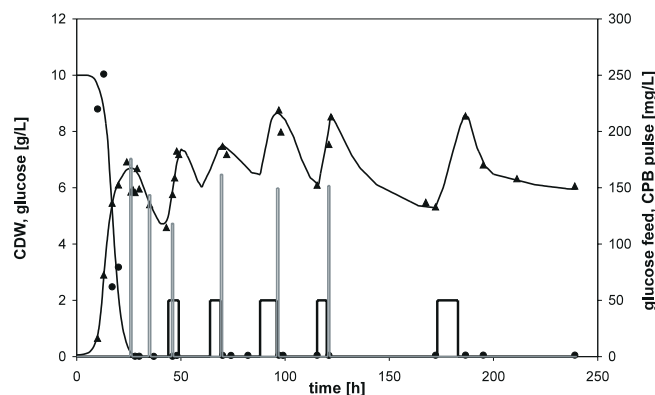
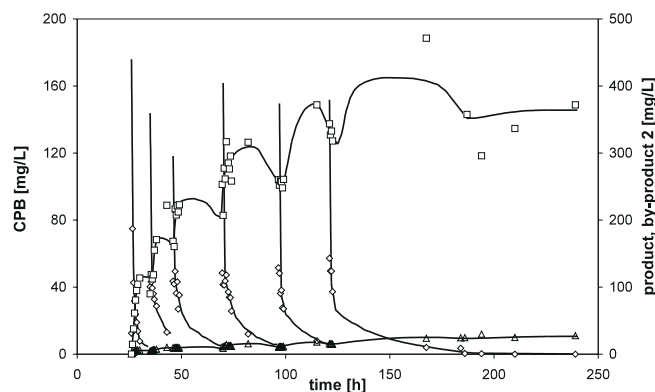


**Fig. 2** Fermentation characteristics when 2-cyclopentyl-1,3-benzoxazole (CPB) is added in the stationary phase. Course of glucose (●), cell dry mass (CDW; ▲), CPB (◇), product (□), by-product 1 (×) and by-product 2 (△)

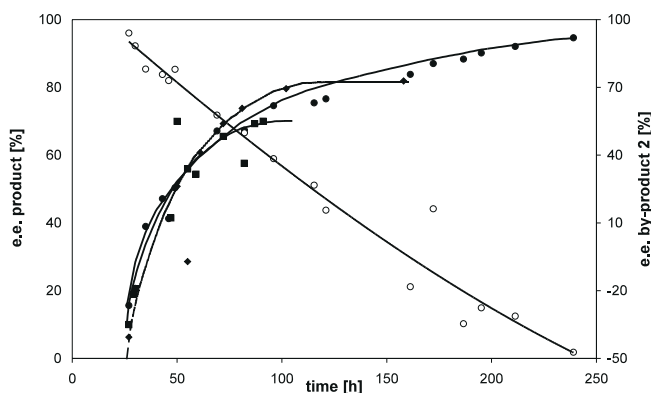


**Fig. 3** 2-Cyclopentyl-1,3-benzoxazole (CPB) fermentation characteristics at different CPB addition times. Course of glucose (●), cell dry mass (CDW; ▲) and product (□), first pulse in the transition phase (19 h, experiment represented with solid symbols) or in the stationary phase (26 h, experiment represented with non-solid symbols)

controlled. The pellets were hollow, indicating transport limitation to the inner hyphae; the pellet size and pH were not reproducible. Literature data indicate that the pH may influence the rate of oxidation of the product to by-product 2 (Schwartz et al. 1996). Therefore, the



**Fig. 4** Fermentation characteristics with biocatalyst regeneration (glucose and pulsed 2-cyclopentyl-1,3-benzoxazole feeding). Course of glucose (●), cell dry mass (CDW; ▲), CPB (◇), product (□), by-product 2 (△). Glucose feed rate, 107 ml h<sup>-1</sup> (black bar) with a concentration of 50 g l<sup>-1</sup>, CPB pulse (open bar)



**Fig. 5** e.e. of product (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol and by-product 2 (3*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-one depending on the substrate feeding profile. Course of the e.e. of the product in the unpulsed process with a 2-cyclopentyl-1,3-benzoxazole (CPB) starting concentration of 350 mg l<sup>-1</sup> (■), in the pulsed process with three pulses (◆), and in the pulsed process with regeneration feed (●) and course of the e.e. of the by-product 2 (○) in the pulsed process with regeneration feed

optimization of the biotransformation process was performed in a bioreactor with controlled pH, aeration, and agitation rate.

Fermentation at an optimal agitation rate of 390 rpm and an inoculum concentration of 10<sup>7</sup> spores l<sup>-1</sup> showed pellet formation in accordance with the literature (Whitaker and Long 1973; Metz and Kossen 1977; Braun and Vecht-Lifshitz 1991).

The pellet size distribution during the cultivation depended mainly on the agitation speed, as the other parameters were kept constant. At a low agitation rate (160 rpm), the fungus grew filamentous. At average and high agitation rates (390, 550 and 730 rpm), loose and fluffy pellets were formed with a maximum diameter of 500 µm providing optimal oxygen transfer to the inner hyphae (Kobayashi et al. 1973; Wittler et al. 1986; Hotop et al. 1993). With increasing agitation rate, the pellet diameter decreased slightly, due to the higher shear forces. However, prevention of pellet formation at high power input, as reported for strains with a coagulative pellet formation (Metz and Kossen 1977), was not found in the investigated range, although pellets did become smaller and more compact.

The stability of the biocatalyst towards the educt CPB decreased significantly at low and at high agitation speeds (Table 1). The surface area of the fungus which was exposed to CPB probably played an important role for the stability of the biomass. Due to the high toxicity of CPB, only low concentrations were tolerated by *C. blakesleeana* (30 mg g<sup>-1</sup> CDW). Although the mechanism of deactivation is not exactly known, a disintegration of cell membranes could be considered, because of the lipophilic character of CPB. The hydroxylation performed via cytochromes *P*-450 is a type of detoxification (Asperger et al. 1999). Filamentous growth (at 160 rpm agitation rate) enhanced membrane surface exposure. Pellet formation probably

protected the cells in the pellet core due to a concentration gradient of CPB from the outer to the inner pellet regions. Presumably, CPB is quickly and strongly absorbed on the outer pellet regions, while the pellet core remains unloaded. At high agitation rates, torn hyphae were observed under the microscope due to mechanical stress. In this damaged state, equivalent amounts of CPB could cause greater harm as confirmed by higher cell death rates (Table 1).

The strong adsorption of CPB on the biomass explains the rapid decrease of its concentration and the low product concentration in the fermentation medium (Kreiner et al. 1996). Extraction of the whole fermentation broth including the biomass will result in higher final product yields. Since representative sampling of the fermentation broth was difficult due to the high amount of solid material, analysis during the fermentation was done from the centrifuged medium in order to obtain reproducible data over all experiments.

The stability of the fungus towards the chemical educt depended on the physiological state of *C. blakesleeana*. The highest stability of the biocatalyst was observed in the stationary growth phase, while addition of CPB in the lag phase or the exponential growth phase of the fungi resulted in very slow conversion rates and rapid cell lyses (Kreiner et al. 1996). However, addition of CPB in the transition phase from the exponential growth phase to the stationary phase did not affect the transformation rate or the enhancement of e.e., but did significantly decrease the stability of the biocatalyst (Fig. 3). This phenomenon may be linked to an altering of the membrane structure due to the increasing age of the fungus (Sedlacek et al. 1981) or due to exposition to toxic agents (Venkateswerlu et al. 1989).

Although the transformation rate was not influenced by variation of the agitation speed, the e.e. decreased with increasing agitation speed (Table 1). Similar to reported observations, by-product 2 was formed by delayed oxidation of the main product (Schwartz et al. 1996). The diastereomers of the main product are presumably oxidized at different rates with slightly faster oxidation of the (1*R*,3*R*)-diastereomer, which could explain the initial enantiomeric pure formation of by-product 2 and the increase of the e.e. of the main product. The regeneration of the fungus with the glucose feed provides active biomass during the whole fermentation, which also increases the e.e. of the main product. The final e.e. is strongly enhanced from 71% to 95%, which represents an increase of 48% over previously reported results (Kreiner et al. 1996).

The optimal strategy for the biotransformation of the toxic educt CPB was a fermentation in optimized medium, at an agitation rate of 390 rpm and an aeration rate of 1 vvm during growth phase and 0.5 vvm during biotransformation. Pulses of CPB started in the stationary phase with 30 mg g CDW<sup>-1</sup>, and the biomass was regenerated with a nutrition feed. Due to this stable semi-continuous process, long-term experiments were performed and the product concentration was strongly

increased while the concentration of the toxic educt CPB was kept low. Further research should be done to clarify the mechanisms of enzymatic e.e. enhancement in order to achieve a pure enantiomeric product.

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