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Regioselective oxidation of terfenadine with *Cunninghamella blakesleeana*

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Abstract

The regioselective oxidation of terfenadine with the fungi *Cunninghamella blakesleeana* was studied as a biochemical alternative for the chemical synthesis of the antihistaminic drug fexofenadine. It was demonstrated that *C. blakesleeana* oxidises the *tert*-butyl group of terfenadine to the corresponding alcohol 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol. A continuous process for regioselective oxidation of terfenadine was developed. Terfenadine was supplied micro-crystalline due to the low solubility in water. Optimum reaction conditions with respect to medium composition, temperature, pH, pO_2, co-substrate and feeding rates were found by means of reaction engineering studies. A cross-flow microfiltration unit was operated in a by-pass of a lab-scale stirred tank reactor for retention of the biocatalysts and the micro-crystalline substrate. The alcohol was continuously removed with the filtrate to minimise product inhibition. Continuous biotransformation of micro-crystalline terfenadine with *C. blakesleeana* in the membrane reactor system with a dilution rate of 33 h at co-substrate concentrations of about 1 up to 3 g/l glycerol in the reactor resulted in a space–time yield of 145 mg of alcohol/l/day and an alcohol yield of 71%. The produced alcohol was easily isolated from the filtrate by adsorption on XAD-4 resin followed by eluation with methanol (concentration factor 7).

Keywords: Terfenadine; *Cunninghamella blakesleeana*; Biotransformation; Micro-crystalline; Membrane reactor

1. Introduction

Terfenadine is a widely used antihistaminic drug with small or no sedative properties. In human liver, terfenadine is almost completely transformed by hepatic cytochrome *P*-450 to the pharmacologically active acid metabolite (fexofenadine) and the inactive metabolite azacyclonol in the first pass [1] (Fig. 1). This hepatic oxidation metabolism is altered by many other drugs, like antifungal (ketoconazole or itraconazole) or antibacterial (clarithromycin or erythromycin) agents [2]. If one of these drugs is consumed together with terfenadine, this antihistamine can accumulate and cause severe cardiovascular side effects [3]. Fexofenadine, the pharmacologically active acid metabolite, is not

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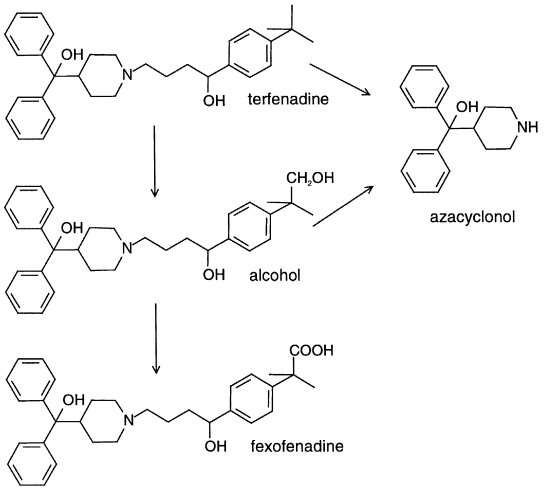

Fig. 1. Hepatic oxidation of terfenadine [1] (alcohol: 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol).

further metabolised by hepatic cytochrome *P*-450. Consequently, fexofenadine was recently introduced as Allegra® to the antihistamine-market avoiding the side effects of terfenadine.

Many reports where published describing the chemical synthesis of fexofenadine [1,2,4,5], which are characterised by laborious reaction techniques and low yields ($<10\%$ in total). The regioselective chemical oxidation of terfenadine as an alternative for the synthesis of fexofenadine has not been described up to now. Schwartz et al. [6] reported the regioselective biochemical oxidation of ebastine — an antihistamine which is quite similar to terfenadine — via microbial oxidation with the filamentous fungi *Cunninghamella blakesleeana*. By means of a batch biotransformation 200 mg/l of ebastine were oxidised and 80 mg/l of the corresponding acid metabolite (carebastine) were produced. It was stated that the same regioselective oxdiation step can be performed by *C. blakesleeana* with terfenadine as substrate [7].

One of the reaction engineering problems of the microbial oxidation of terfenadine is the low substrate solubility in water (~ 20 mg/l). This limits the availability of the substrate for the biocatalyst [8]. Another issue, which has to be considered, is the selection and addition of a suitable co-substrate for cofactor regeneration inside the cells catalysing the oxidation of terfenadine [9]. Last but not least, increasing product concentration in the reactor may result in product inhibition. In case of inhibitory product concentration levels in the reactor, a simultaneous product removal may be beneficial to improve process performance.

The present work deals with reaction engineering studies and the development of a biotransformation process for regioselective oxidation of terfenadine with *C. blakesleeana*. Most of all, reaction engineering problems like low substrate solubility in water, co-substrate addition for cofactor regeneration and product inhibition are addressed.

2. Material and methods

2.1. Organism

C. blakesleeana DSM 1906 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Stock cultures of the organism were maintained on potato-dextrose (PD) agar slants, stored at 4°C and subcultured for 4 days every 4 weeks at 30°C.

2.2. Media

The fermentation-medium consisted of (g/l): 20.0 glucose, 12.0 $(NH_4)_2SO_4$, 1.4 K_2HPO_4, 2.5 $MgSO_4 7 \cdot H_2O$ and (mg/l) 20.0 EDTA, 200.0 $CaCl_2 \cdot 2\ H_2O$, 100.0 $MgCl_2 \cdot 6\ H_2O$, 28.0 $FeSO_4 \cdot 7\ H_2O$, 4.0 $MnCl_2 \cdot 4\ H_2O$, 3.8 $ZnCl_2$, 0.27 $CuCl_2 \cdot 6\ H_2O$, 0.02 $CoCl_2\ 6H_2O$ and 0.06 $Na_2MoO_4 \cdot 2\ H_2O$.

The fermentation-medium was sterilised without glucose at 121°C for 20 min. Separately sterilised glucose solution was added afterwards. In order to avoid foaming 200 μl/l of sterile antifoam solution (Sigma #289) was added aseptically to the fermentation-medium.

2.3. Inoculum-preparation

C. blakesleeana was cultivated on 30-ml PD agar in a 500-ml Erlenmeyer-flask at 30°C. After 4 days 170 ml of sterilised fermentation-medium was added to the flask. The incubated PD agar and the fermentation medium were homogenised by means of a dispergation-tool (IKA-Ultra-Turrax T-25 basic with head S25N-18G, IKA-laboratories, Germany) within 50 s at a speed of 28.000 rpm. Shake flasks and the stirred tank reactor were inoculated with 10% (v/v) of this solution.

2.4. Stirred tank reactor

Biotransformations were performed in a stirred tank reactor (KLF 2000, Bioengineering, Switzerland) with flat-bladed disk turbines without baffles and a working volume of 1.7 l. The fermentation unit was equipped with standard measuring and control units (temperature, pressure, pH, pO_2, stirrer speed, and weight). The pH set-point of 6.5 was controlled by addition of 2 N NaOH or 2 N H_2SO_4, respectively. Dissolved oxygen concentration was measured with a clark-type electrode (Ingold, Germany). The pO_2 set-point of 60% air saturation at atmospheric pressure was controlled by varying the air flow rate between 0 and 500 ml/min (0–0.3 vvm) at a constant stirrer speed of 1200 rpm. Growth of *C. blakesleeana* and biotransformation were performed at 30°C.

Fermentation medium was fed into the reactor with a high-precision piston pump (Dosimat 665, Methrom, Switzerland) at low feed rates (300–50000 μl/h) and with a peristaltic pump (WM 501U, Watson-Marlow, UK) at high feed rates (50–1200 ml/h). The peristaltic pump was controlled gravimetrically (YFC02Z, Satorius, Germany).

For cell retention in a continuous process, a by-pass was connected to the stirred tank reactor. The by-pass consisted of a peristaltic pump (WM 604, Watson-Marlow) and a cross-flow microfiltration-unit (MD 020 TP 2N, Microdyn, Germany) with three tubes made of polypropylene with an inner diameter of 5.5 mm, a pore size of 0.2 μm and an interfacial area of 0.036 m^2. A flow-rate of 2.1 l/min was kept constant in the by-pass.

2.5. Standard cultivation in the stirred tank reactor

A total of 1.4 l of fermentation medium were in-situ sterilised at 121°C for 20 min without glucose, while 34 g of glucose solved in 100 ml of water were sterilised separately and added aseptically. Batch growth of *C. blakesleeana* was performed after the addition of the inoculum (10% v/v). Two further additions of 34 g of glucose solved in 100 ml of water were performed at a process time of 25 h and of 29 h after inoculation. The previously added glucose was completely consumed each time glucose was added. At a process time of 33 h, just before the glucose in the reactor was depleted, 17 g of glycerol solved in 50 ml of water were added. Due to the sampling, the volume in the reactor was not changed significantly.

2.6. Analytical methods

For determination of dry cell mass, 2 ml of culture broth were centrifuged (5 min at $5000 \times g$) in a pre-weighted polypropylene test tube. After removal of the supernatant the pellet was washed with water and the solution was again centrifuged (5 min at $5000 \times g$). The supernatant was removed, the test tube was dried at 80°C for 48 h and dry cell mass was determined gravimetrically.

Concentrations of terfenadine and the corresponding alcohol were determined by means of HPLC: A sample with the cell-suspension was diluted 50% by addition of methanol. After vigorous mixing for 30 s the suspension was centrifuged (5 min at $5000 \times g$). The supernatant was diluted with 50% methanol (v/v) until maximum concentrations of 10 mg/l of terfenadine or the corresponding alcohol were

achieved. The diluted sample was analysed with reversed phase HPLC (50% acetonitrile, 1% TFA; flow: 1 ml/min; column: LiChrosphere 100 RP 18 EC-5, 4.6 × 250 mm; detection: fluorescense (extinction: 230 nm, emission 295 nm); retention-times: terfenadine 14.5 min, alcohol 6.2 min).

2.7. Identification of the oxidation product

The biotransformation product was extracted in diethylether and purified by silica gel chromatography with $CHCl_3$:MeOH:HAc (85:15:1) as eluent. For identification of the purified oxidation product, FAB-MS and NMR-spectroscopy were applied.

2.8. pH-studies for biotransformation

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. The fermentation broth was harvested after a process time of 45 h. Each of parallel operated 1000 ml fed-batch shake flasks were filled aseptically with 100 ml of the cell-suspension. The fed-batch shake flasks were incubated at 150 rpm and 30°C in a pH-controlled fed-batch shake flask system (fedbatch-pro, DASGIP, Juelich, Germany) [10]. Within 2 h, the pH in the individual fed-batch shake flasks was controlled to preset set-points of pH 5.0, pH 5.75, pH 6.5, pH 7.25, and pH 8.0, respectively. After an incubation of 1 h at the individual pH set-points, 20 mg of terfenadine solved in 100 μl of dimethylformamide (DMF) was added to the suspension. The produced alcohol-concentration was determined after a process time of 27 h.

2.9. Substrate addition studies for biotransformation

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. With a step time of 4 h, samples of 10 ml each were withdrawn and aseptically transferred into 100 ml shake flasks. After addition of 2 mg of terfenadine solved in 100 μl of DMF, the shake flasks were incubated at 30°C and 150 rpm. The produced alcohol concentrations in the individual shake flasks were determined after a process time of 48 h.

2.10. Screening for co-substrates

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. The fermentation broth was harvested after a process time of 60 h. Each of parallel operated 100 ml shake flasks were filled aseptically with 10 ml of the cell-suspension and individual co-substrates (4 g/l). After addition of 150 mg/l of terfenadine the flasks were incubated at 30°C and 150 rpm. The resulting alcohol concentrations were analysed after a process time of 48 h.

2.11. Experimental design for glycerol, salts and terfenadine addition

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. The fermentation broth was harvested after a process time of 50 h. Each of 27 parallel operated 500 ml shake flasks were filled aseptically with 50 ml of the cell-suspension. Glycerol, salts and terfenadine were added according to a statistical design. A central composite design was used for the estimation of the parameters of a full second-order polynomial model with four factors for estimation of product concentration [11].

The factors varied within this experiment were: (i) the total amount of glycerol added, (ii) the amount of salts added, (iii) the number of individual additions of glycerol and salts, and (iv) the number of individual additions of terfenadine (see Table 1). The total amount of terfenadine added was fixed to 800 mg/l (40 mg terfenadine in 400 μl DMF).

The parameter of the full second-order polynomial model were estimated by minimising the estimation error using the software Mathematica

Table 1
Experimental design for studies on product formation (terfenadine oxidation with *C. blakesleeana*) as function of glycerol, salts, and terfenadine addition applying a central composite design for the estimation of the parameters of a full second-order polynomial model with four factors (factor 1: number of additions of terfenadine with a total amount of 800 mg/l; factor 2: total amount of added glycerol (g); factor 3: total amount of added mineral salts with respect to the initial amount; factor 4: number of additions of glycerol and mineral salts)

Run #	Factor 1	Factor 2	Factor 3	Factor 4
1	2	1	1/4	2
2	8	1	1/4	2
3	2	3	1/4	2
4	8	3	1/4	2
5	2	1	1/64	2
6	8	1	1/64	2
7	2	3	1/64	2
8	8	3	1/64	2
9	2	1	1/4	8
10	8	1	1/4	8
11	2	3	1/4	8
12	8	3	1/4	8
13	2	1	1/64	8
14	8	1	1/64	8
15	2	3	1/64	8
16	8	3	1/64	8
17	1	2	1/16	4
18	16	2	1/16	4
19	4	0	1/16	4
20	4	4	1/16	4
21	4	2	1	4
22	4	2	0	4
23	4	2	1/16	1
24	4	2	1/16	16
25	4	2	1/16	4
26	4	2	1/16	4
27	4	2	1/16	4

3.0 (Wolfram-Research, USA). Non-significant parameters with a *p*-value of more than 0.05 were eliminated one after the other.

2.12. Biotransformation in the stirred tank reactor

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. The cells were induced by addition of 30 mg/l of terfenadine at a process time of 38 h. Starting at a process time of 44 h, intermittend addition of 1 g of terfenadine solved in 10 ml of DMF was performed in a way that the terfenadine concentration in the reactor was kept between 30 and 100 mg/l throughout the whole process. The co-substrate glycerol was added with a step time of 12 h (6.3 g/l/day).

2.13. Continuous biotransformation with cell-retention

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor. At a process time of 30 h, the operation of by-pass with the cross-flow microfiltration unit with 3 tubes made of polypropylene was initiated. The cells were induced by addition of 30 mg/l of terfenadine at a process time of 38 h. Starting at a process time of 44 h, intermittend addition of 1 g of terfenadine solved in 10 ml of DMF was performed in a way that the terfenadine concentration in the reactor was kept between 30 and 100 mg/l throughout the whole process. At a process time of 64 h, the co-substrate supply (6 g/l of glycerol solved in fermentation medium without glucose, reduced salt contend (factor 0.15), and 50 mM NaCl) was started with a feed rate of 53 ml/h. The liquid volume in the reactor system was kept constant by controlling the filtrate flow rate.

To perform a biotransformation without co-substrate limitation, the glycerol concentration in the feed-medium was increased to 25 g/l.

3. Results and discussion

3.1. Identification of the biotransformation product

Regioselective oxidation of terfenadine with *C. blakesleeana* was first performed using the reaction conditions described by Schwartz et al. [6] for the oxidation of ebastine. The resulting product was extracted in diethylether and purified by silica gel chromatography with $CHCl_3$:MeOH:HAc (85:15:1) as eluent. The extract was identified by FAB-MS and NMR-

spectroscopy as the corresponding alcohol of terfenadine (see Fig. 1) FAB-MS: $m/z = 488.0$ $(M + H)^{\oplus}$, 470.0 $(M-H_2O)^{\oplus}$, 280.0 $(R_2N{=}CH_2)^{\oplus}$, 183.0 $(Ph_2C{=}OH)^{\oplus}$ ^{1}H NMR (400 MHz, CD_3OD, 25°C): $\delta = 1.28$ (s, 6H), 1.74 (m, 8H), 2.83 (t, 1H), 2.97 (m, 2H), 3.05 (t, 2H), 3.49 (m, 2H), 3.54 (s, 2H), 4.66 (t, 1H), 7.17 (t, 2H), 7.29 (m, 6H), 7.36 (d, ^3J(H,H) = 7.4, 2H), 7.51 (d, ^3J(H,H) = 7.4, 4H).

3.2. Identification of a fermentation medium

Different fermentation media were tested in shake flask experiments for the growth of *C. blakesleeana* and their ability to ensure the regioselective oxidation of terfenadine. Promising results were achieved with a defined mineral salts medium originally developed for *Bacillus subtilis* [12]: About 60% of the product yield of the complex medium used by Schwartz et al. [6] was achieved with this medium. For further improvement of the defined medium *C. blakesleeana* was grown in the complex medium and harvested in the exponential growth. An elemental balance based on the elemental analysis of the composition of *C. blakesleeana* revealed a lack of nitrogen, calcium, sulphur and magnesium in the defined medium. Supplementation of these elements by addition of the corresponding mineral salts resulted in the same process performance with respect to growth and terfenadine oxidation, which has been achieved with the complex medium used before.

The addition of soybean-peptone for induction of cytochrome *P*-450 as described by Schwartz et al. [13] and Sariaslani and Kunz [14] was found not to be necessary. The identified defined mineral salt medium was used for all further biotransformation studies.

3.3. Improvement of substrate availability

Different approaches to overcome the low solubility of terfenadine in water were studied in shake-flasks and the lab-scale stirred tank reactor:

1. Enhancing substrate solubility by addition of water solubility mediators.
2. Adding a non-water miscible organic phase with solved terfenadine.
3. Reducing mass transfer limitations by micro-crystalline terfenadine addition.

Solubility mediators tested were Polyethylenglycol (PEG) 600, and PEG 10000, Polyvinylpyrrolidon K-15, and Polyvinylpyrrolidon K-30. Best biotransformation results were observed in shake flask studies, if 10% (w/v) PEG-600 was added to the medium. Unfortunately, scale-up into the stirred tank reactor was not successful: Low biotranformation rates were measured (24 mg of alcohol/l/day). Moreover, the viscosity of the *C. blakesleeana* cell suspension in the stirred tank reactor increased dramatically compared to the shake flask.

Shake flask studies were also performed with additions of non-water miscible organic solvents as a substrate reservoir for terfenadine [15]. It was found, that solvents with a high solution capacity for terfenadine were toxic to the the *C. blakesleeana* cells (e.g. 1-octanol, diphenylether, 2-undecanon, *p*-xylene). The only non-toxic solvent tested with a moderate solubition capacity for terfenadine was 1-butylstearate. Unfortunately, this organic solvent was consumed by *C. blakesleeana*.

Micro-crystalline terfenadine addition was found to be the most effective method to achieve high oxidation rates with *C. blakesleeana.* Micro-crystalline terfenadine can be achieved by solving terfenadine in a water miscible solvent like methanol, ethanol, DMF or dimethylsulfoxid (DMSO). This solution must be injected into the fermentation medium in such a way, that a fast mixing of the water miscible solvent with the solved terfenadine is assured. This results in an immediate crystallisation of terfenadine in the aqueous fermentation medium at any position in the reactor, where the water miscible solvent concentration is reduced. Mi-

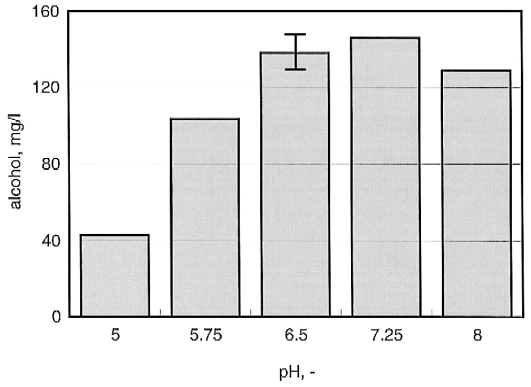

Fig. 2. Terfenadine oxidation with *C. blakesleeana* in fed-batch shake flasks: measured alcohol concentrations (1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol) at a process time of 24 h as function of pH set-points.

cro-crystals with a high surface-to-volume ratio are formed. The solvent with the solved terfenadine can be easily sterilised by microfiltration before injection into the fermentation broth.

3.4. Reaction conditions: temperature, oxygen tension and pH

Shake flask studies were performed to compare the regioselective oxidation of terfenadine with *C. blakesleeana* at temperatures between 21°C and 37°C. Best biotransformation rates were achieved at 30°C. The application of a temperature of 30°C was reported for other biotransformations with *C. blakesleeana* as well [16].

The study of pO_2-levels in the range of 5% up to 80% oxygen saturation at atmospheric pressure in the stirred tank reactor indicated a minimum pO_2 of 20% air saturation. Decreasing the oxygen tension below this level resulted in a decrease of the biotransformation rate.

The decrease of biotransformation activity at oxygen concentrations below 20% air saturation in the reactor may be caused by oxygen transport limitations into the pellets of *C. blakesleeana.*

Comparing the process performance in pH-controlled shake flasks demonstrates that highest alcohol concentrations were achieved at pH 6.5 and pH 7.25 (see Fig. 2). A set-point of pH 6.5 was chosen, because the growth rate of *C. blakesleeana* was higher at pH 6.5 compared to pH 7.5.

3.5. Initiation of the biotransformation

C. blakesleeana cells were produced via the standard cultivation in the stirred tank reactor (see Fig. 3). The biotransformation activity of the cells was measured with a step time of 4 h, applying a standardised activity test in shake flasks. Maximum biotransformation activities of the *C. blakesleeana* cells were observed at a process time of about 50 h ($\pm$10 h) (see Fig. 4). At a process time of 50 h, the glucose in the stirred tank reactor was totally depleted and the glycerol concentration was low (see Fig. 3).

As in the case of *B. megaterium* [17], a maximum oxidative biotransformation activity of *C. blakesleeana* was observed in the early stationary cultivation period. The following de-

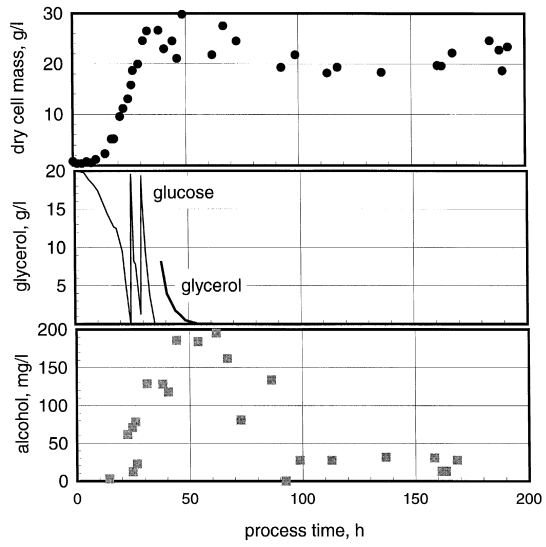

Fig. 3. 'Standard' cultivation of *C. blakesleeana* in a stirred tank reactor: Substrate concentrations (glucose and glycerol), dry cell mass and biotransformation activity of *C. blakesleeana* cells (measured by means of activity tests in shake flasks) as function of process time (alcohol: 1-[4-(1,1-dimethyl-2-hydroxyethyl) phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol).

crease in biotransformation activity may be a consequence of a lack of reduction equivalents or an inactivation of the catalyst.

3.6. Screening for co-substrates

The incubation of *C. blakesleeana* cells produced via the standard cultivation in the stirred tank reactor with individual co-substrates and terfenadine in shake flasks resulted in a maximum alcohol production, if glycerol is used as co-substrate (see Fig. 4). A few other co-substrates (hexadecane, starch, citrate) had no significant effect on the terfenadine oxidation activity. All other co-substrates resulted in a decreased alcohol production.

3.7. Identification of glycerol, salts and terfenadine additions

Shake flask studies were performed with *C. blakesleeana* cells produced via the standard cultivation in the stirred tank reactor. Glycerol, salts and terfenadine were added into the parallel operated shake flasks according to a central composite design.

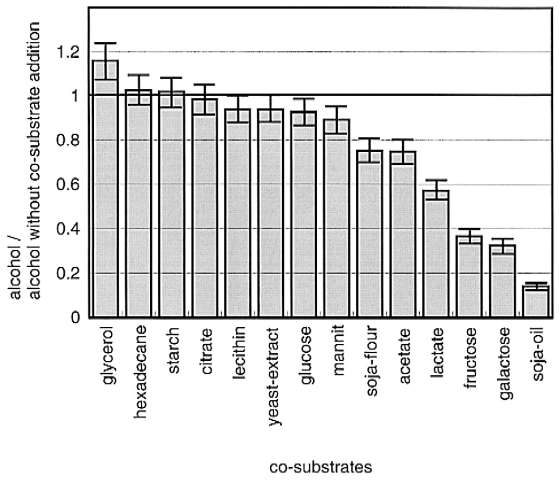

Fig. 4. Co-substrate screening for terfenadine oxidation with *C. blakesleeana*: produced alcohol (1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol) after an incubation of 48 h in shake flasks (reference: no co-substrate addition).

Table 2
Identified normalised parameters of a full second-order polynomial model with four factors for estimation of product concentration (terfenadine oxidation with *C. blakesleeana*) as function of (factor 1) the number of additions of terfenadine with a total amount of 800 mg/l, (factor 2) the total amount of added glycerol (g), (factor 3) the total amount of added mineral salts with respect to the initial amount, and (factor 4) the number of additions of glycerol and mineral salts (non-significant parameters with a *p*-value of more than 0.05 were eliminated one after the other)

Parameter	Value	*p*-Value
a_0	240	$<1\times10^{-9}$
a_1	−34	1×10^{-8}
a_{11}	−18	1×10^{-4}
a_2	−14	3×10^{-4}
a_{22}	−23	3×10^{-8}
a_4	17	4×10^{-4}
a_{24}	12	2×10^{-2}

The measured alcohol concentrations were used for estimation of the parameters of a full second-order polynomial model with four factors (see Table 2). The identified polynomial model was able to describe 88% of the total variance. The observed variance of the individual shake flask experiments was about 8%.

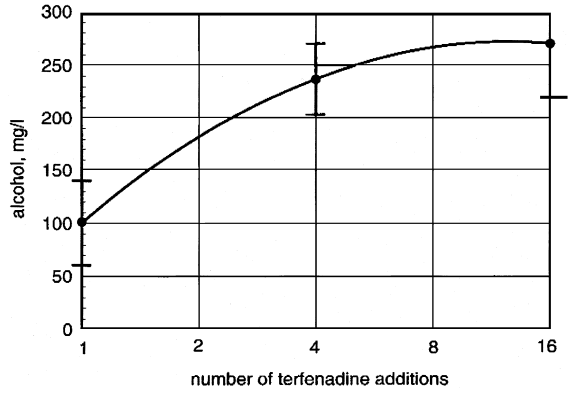

Fig. 5. Terfenadine oxidation with *C. blakesleeana*: Measured and estimated product concentration as function of the number of additions of terfenadine within a period of 120 h (total amount of terfenadine: 800 mg/l). Estimation based on the identified second-order model of the alcohol concentration as function of the number of terfenadine additions, total amount of glycerol, total amount of mineral salts, and the number of glycerol and salt additions (alcohol: 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol).

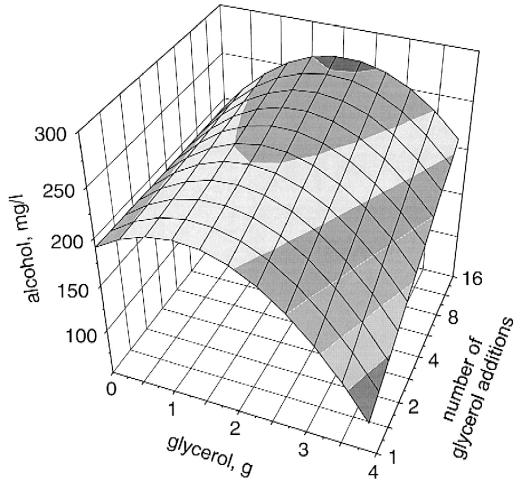

Fig. 6. Terfenadine oxidation with *C. blakesleeana*: Estimated product concentration as function of the total amount of glycerol and the number of glycerol additions within a period of 120 h. Estimation based on the identified second-order model of the alcohol concentration as function of the number of terfenadine additions, total amount of glycerol, total amount of mineral salts, and the number of glycerol and salt additions (alcohol: 1-[4-(1, 1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl) 1-piperidinyl]-1-butanol).

It was demonstrated that the resulting alcohol concentration is a function of the number of terfenadine additions: Biotransformation of terfenadine was improved, if the total amount of terfenadine was added in small amounts with higher step times, instead of one initial addition (see Fig. 5).

This 'micro-crystalline substrate inhibition' effect may be caused by agglomeration of micro-crystals, thus reducing the interfacial area and the substrate availability. About 24 h after the initial addition of the total terfenadine amount into the shake flasks crystals of terfenadine were observed at the glass-wall above the circulating bulk liquid. This part of the substrate was no longer accessible for the biotransformation.

Based on these results, it can be concluded that terfenadine should be added in small amounts which result in terfenadine concentrations of about 50 up to 100 mg/l in the fermentation broth (see Fig. 5).

The simulation of the estimated alcohol concentrations as function of the added glycerol amount and the number of glycerol additions indicates (see Fig. 6): The total amount of glycerol should be 2 g on the shake flask scale with 16 individual additions within 120 h. This correspondents to an intermittend glycerol feed rate of 6 g/l/day. Regioselective oxidation of terfenadine is low, if a high amount of glycerol is added with one initial addition. It seems that glycerol inhibits the biotransformation at high concentrations.

No significant effects were observed with respect to the addition of mineral salts (the *p*-value of the corresponding parameters were more than 0.05, see Table 2).

3.8. Regioselective terfenadine oxidation in a fed-batch process

The identified reaction conditions for terfenadine oxidation with *C. blakesleeana* were applied for performing the biotransformation in a lab-scale stirred tank reactor with intermittend feeding of terfenadine and glycerol (see Fig. 7). An alcohol concentration of 600 mg/l was

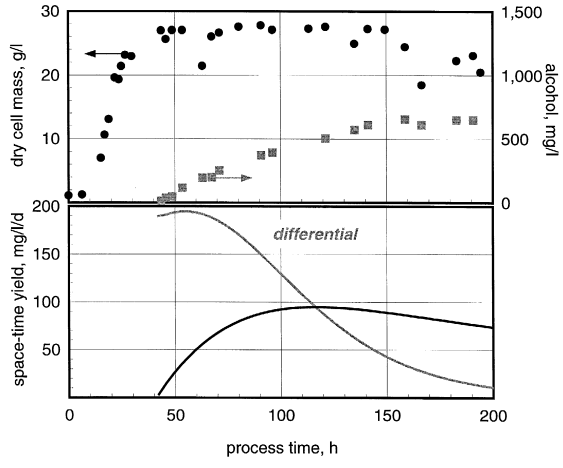

Fig. 7. Regioselective oxidation of terfenadine with *C. blakesleeana* in a fed-batch process: dry cell mass concentration, alcohol concentration (1-[4-(1,1-dimethyl-2-hydroxyethyl)-phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol) and space–time yields as function of process time.

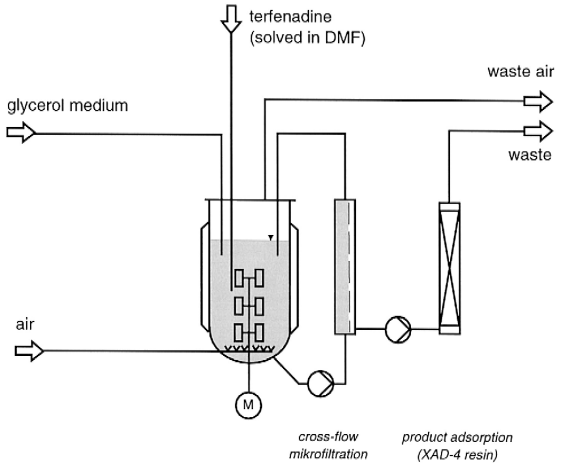

Fig. 8. Scheme of the membrane reactor system for continuous biotransformation of micro-crystalline terfenadine with *C. blakesleeana*.

achieved within a process time of 200 h. The total amount of alcohol produced was 1 g. This correspondents to a space–time yield of 72 mg of alcohol/l/day. The differential space–time yield with an initial alcohol production rate of 190 mg of alcohol/l/day decreases with increasing process time. At the end of this biotransformation process, no further terfenadine oxidation was observed. Additional supply of glycerol did not restore the biotransformation activity of the *C. blakesleeana* cells in the reactor (data not indicated). It is concluded that an alcohol concentration of 600 mg/l in the stirred tank reactor totally inhibits the biotransformation.

Terfanadine was oxidised to the corresponding alcohol with a yield of 81%. At the end of the process, the differential selectivity decreased down to 35%. This decrease may be caused by a further degradation of the biotransformation product at higher alcohol concentrations in the stirred tank reactor. However, no other oxidation products were detected by HPLC-analysis. Further investigations with labelled terfenadine may be useful to identify by-products or degradation products (see for example Ref. [18]).

A consequence of the observed product inhibition from the reaction engineering point of view may be a simultaneous product removal to ensure low alcohol concentrations in the stirred tank reactor.

3.9. Continuous biotransformation with cell-retention

The integration of a cross-flow microfiltration unit in the by-pass of a continuous operated stirred tank reactor (see Fig. 8) for retention of the cells and the micro-crystalline terfenadine allowed the removal of all soluble compounds including the biotransformation product. Solved terfenadine (< 20 mg/l) could pass the membrane too.

The continuous product removal at a dilution rate of 33 h resulted in an alcohol concentration of about 200 up to 250 mg/l in the membrane reactor system (see Fig. 9). The total amount of alcohol produced within a process time of 200 h was 1.6 g. The resulting space–time yield (105 mg of alcohol/l/day) was improved by 45% compared to the fed-batch process. The decrease of the differential space–time yield with the same initial alcohol production rate as before (190 mg of alcohol/l/day) was less severe

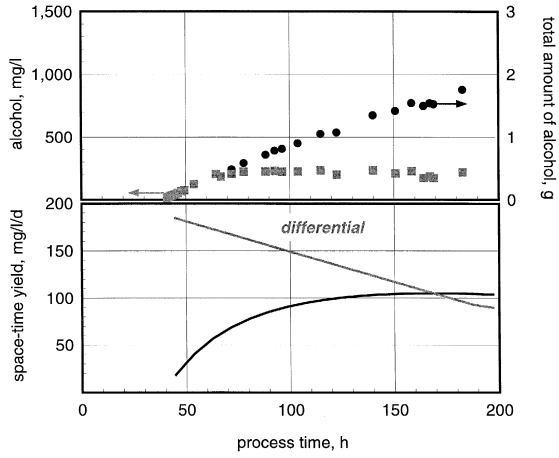

Fig. 9. Continuous oxidation of terfenadine with *C. blakesleeana* in a membrane reactor system (dilution rate 33 h, glycerol in the feed 6 g/l): alcohol concentration, total amount of the produced alcohol and space–time yields as function of process time (alcohol: 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol).

compared to the fed-batch process. At the end of biotransformation process, a differential space–time yield of 80 mg of alcohol/l/day was measured.

Terfanadine was oxidised to the corresponding alcohol with a yield of 72%. To achieve a further process integration (biotransformation and product isolation) the filtrate was directly fed onto a 500-ml XAD-4 resin column (Fig. 8). This column allowed complete retention of the produced alcohol throughout the whole continuous biotransformation process. Pure methanol was used for elution of the adsorbed alcohol. A concentration factor of 7 was achieved (1.75 g of oxidation product/l methanol).

3.10. Continuous biotransformation with excess of glycerol

To investigate the effect of the co-substrate glycerol on the differential space–time yield, the same continuous biotransformation process was performed in the membrane reactor system with a glycerol-content of the feed medium of 25 g/l instead of 6 g/l. The resulting glycerol concentration in the reactor system was about 1 up to 3 g/l.

The continuous product removal at a residence-time of 0.03 1/h resulted in an alcohol concentration of about 250 mg/l with a yield of 71% in the membrane reactor system (see Fig. 10). The total amount of alcohol produced within a process time of 240 h was 2.7 g. The resulting space–time yield (145 mg of alcohol/l/day) was improved by 100% compared to the fed-batch process. No decrease of the differential space–time yield was observed within the estimation error.

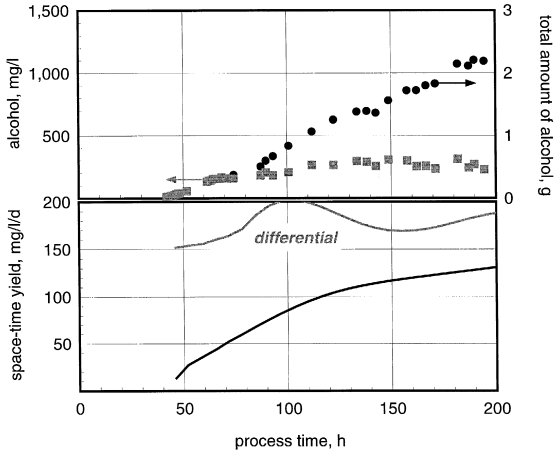

Fig. 10. Continuous oxidation of terfenadine with *C. blakesleeana* in a membrane reactor system with increased co-substrate supply (dilution rate 33 h, glycerol in the feed 25 g/l): alcohol concentration, total amount of the produced alcohol and space–time yields as function of process time (alcohol: 1-[4-(1,1-dimethyl-2-hydroxyethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-1-butanol).

4. Conclusions

It was demonstrated, that the fungi *C. blakesleeana* was not able to perform the regioselective oxdiation of terfenadine to the pharmacologically active acid metabolite fexofenadine. However, the regioselective activation of the *tert*-butyl group of terfenadine with *C. blakesleeana* yielding the corresponding alcohol in presence of the other functional groups of terfenadine offers the chance for a simple chemical synthesis of fexofenadine [5]: The alcohol could be oxidised with strong agents (e.g. $RuCl_3$ 5 H_2O with H_5IO_6), but the secondary hydroxy group of the biotransformation product will be oxidised as well. Therefore, a selective reduction of this group by $NaBH_4$ is necessary to gain fexofenadine.

Based on reaction engineering studies, a biotransformation process was developed for regioselective oxidation of terfenadine to the corresponding alcohol with *C. blakesleeana*. The lack of terfenadine availability due to the low substrate solubility in water was solved by micro-crystalline terfenadine addition. The biotransformation was performed continuously with a cross-flow microfiltration unit in the by-pass of a stirred tank reactor to overcome product inhibition. Retention of the *C. blakesleeana* cells and the micro-crystalline terfenadine in the

membrane reactor system and product concentrations far below the level of total inhibition of the biotransformation, as well as a sufficient supply of the co-substrate glycerol, resulted in a 100% increase of the alcohol space–time yield compared to the fed-batch process.

This membrane reactor configuration with micro-crystalline substrate supply may be, in general, a suitable reactor system for biotransformations which are characterised by (i) low substrate solubility in water, (ii) improved product solubility in water compared to the substrate, and (iii) product inhibition [19].

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