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Influence of Additives on the Biohydroxylation of Protected Carboxylic Acids and Ketones

Michaela Kreiner[1], *Gerhart Braunegg*[1]*, *Anna de Raadt*[2], *Herfried Griengl*[2], *I. Kopper*[1], *O. Sukcharoen*[1] *and H. Weber*[2]

[1]Institute of Biotechnology, Technical University of Graz, Petersgasse 12, 8010 Graz, Austria, Spezialforschungsbereich F01 Biocatalysis
[2]Institute of Organic Chemistry, Technical University of Graz, Stremayrgasse 16, 8010 Graz, Austria, Spezialforschungsbereich F01 Biocatalysis

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Summary

The effects of β-cyclodextrin (β-CD), the anionic detergent Witconate SK, two non-ionic surfactants, Triton X-100 and Tween 80, and sodium tetraborate on the biohydroxylation of protected cyclic carboxylic acids and ketones by Bacillus megaterium and the fungi Beauveria bassiana, Curvularia lunata, and Cunninghamella blakesleeana were studied in shake flask experiments.

The bacterium B. megaterium was found to be more sensitive towards the above mentioned surfactants than the fungi tested. The presence of Triton X-100 (0.5 fold CMC) and Tween 80 (0.5–2.0 g L^{-1}) decreased the maximum concentration of the main hydroxylated product by 62% and 55%, respectively. Employing Witconate, hydroxylation was not observed at all. Only β-CD (0.5 g L^{-1}) proved to be slightly beneficial for the transformation using B. megaterium demonstrated by a 5% increase of the overall yield.

Contrary to the results obtained with B. megaterium, the fungi under consideration showed an improvement, with respect to the biotransformation with all the additives tested. Generally low detergent concentrations (up to 1 g L^{-1}) were preferable. Most positive results for the transformation with C. lunata were found with 0.5 g L^{-1} β-CD and 1 g L^{-1} Tween 80 allowing a 65% increase of the maximum yield of the main hydroxylated product. The presence of 12 mg L^1 sodium tetraborate increased the maximum concentration of the main product when employing C. blakesleeana. For B. bassiana, a 33% increase of the maximum product concentration was obtained in the presence of Witconate (0.5 and 1.0 g L^{-1}).

Keywords: biohydroxylation, β-cyclodextrin, *B. megaterium, B. bassiana, C. lunata, C. blakesleeana,* surfactants, cyclic carboxylic acids and ketones

Introduction

Biocatalytic reactions are increasingly used in organic synthesis due to the regio- and stereoselectivity exhibited by enzymic reactions *(1,2)*. The feasible application of such a bioprocess requires a high concentration of products and fast kinetics. However, the efficiency of biocatalysis with whole cells is often seriously impeded by the poor bioavailability of lipophilic substrates. Attempts to overcome these shortcomings include the use of detergents *(3–5)*, organic cosolvents *(6,7)*, cyclodextrins *(5,8–13)*, polymers *(14)*, and liposomal *(15)* or aqueous biphasic systems *(16)*.

Surfactants are able to enhance the bioavailability of hydrophobic compounds because these molecules tend to concentrate at surfaces as well as interfaces and therefore decrease the levels of surface tension and interfacial tension. Consequently this leads to the dispersion of hydrophobic molecules *(17)*. Another important characteristic of surfactants is the fact that, above a certain detergent concentration (critical micelle concentration = CMC), aggregates (micelles) are formed. If the system contains lipophilic substances, these are solubilized by inclusion into the hydrophobic cores of the micelles. As

* corresponding author

a consequence, the apparent solubility of almost insoluble chemicals is strongly enhanced above the CMC.

Cyclodextrins (CDs) are nonreducing cyclic oligomers of 1,4-α-linked D-glucose units enzymatically produced from starch. The main naturally occurring CDs consist of six, seven, or eight glucose molecules, forming α-, β-, and γ-CDs, respectively. The molecular structure of CDs is a cylinder-like shape, with a hydrophilic shell and an apolar internal cavity. A consequence of this structure is the ability to accommodate suitably sized organic guest molecules in the cavity, thus forming inclusion bodies *(8,18)*. This clathrating ability of CDs offers the additional benefit of a reduction of the possible toxicity effects exerted by the organic substrate/product molecules on the cells. Consequently CDs are increasingly being used for enhancing the bioavailability of organic substrates to biocatalysts *(5,8–13)*.

In this study, we investigated the effects of β-CD, one anionic and two non-ionic surfactants as well as of sodium tetraborate on the rates of product formation and maximum product concentrations. *B. megaterium* and three fungi, which are widely used for biocatalytic processes *(1,2)*, *B. bassiana*, *C. lunata* and *C. blakesleeana*, were chosen as microorganisms.

2-Cycloalkylbenzoxazoles (**1a**) and (**2a**) and 4-*N*-benzoyl-1-oxa-4-aza-spiro[4.4]-nonane (**3a**) were used as substrates for these biotransformations (Table 1). These benzoxazoles (**1a** and **2a**) and the spirooxazolidine (**3a**) serve as nonpolar substitutes for cyclic carboxylic acids and ketones, respectively. In this manner degradation of the respective functional group by the microbial cells is prevented *(19)*.

Materials and Methods

Strains and cultivation. *Cunninghamella blakesleeana* DSM 1906 was obtained from DSM, Braunschweig, Germany; *Curvularia lunata* CBS 21554 from CBS, Baarn, the Netherlands; *Beauveria bassiana* ATCC 7159 from ATCC, Rockeville, USA; and *Bacillus megaterium* CCM 2037 (non sporulating mutant KM) from CCM, Brno, Slovakia. Stock cultures of the first two strains were maintained on potato-dextrose (PD) agar slants, the other two strains were maintained on modified medium E1 agar slants. The cultures were stored at 4 °C and subcultured every three weeks at 30 °C (*B. bassiana* at 24 °C).

Media. Medium E, modified medium E1, and Czapek-Dox medium were prepared as described by Kreiner *et al. (21)*. Modified medium E4 consisted (per L) of 5 g of peptone, 2 g of yeast extract (Oxoid), 20 g of malt extract, and 10 g of glucose. The glucose-mineral salt medium for *B. megaterium* consisted of (per L) 4.5 g of Na_2HPO_4 2 H_2O, 1.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, 0.05 g of Fe(III)NH_4citrate (17%), 0.02 g of $CaCl_2 \cdot 2\ H_2O$, 20 g of glucose, 3 g of $(NH_4)_2SO_4$, 1 g of sodium-acetate, 1 g of yeast extract, and 1 mL of trace element solution SL-6. Solution SL-6 consisted (per L) of $ZnSO_4 \cdot 7\ H_2O$ (100 mg), $MnCl_2 \cdot 4\ H_2O$ (30 mg), H_3BO_3 (300 mg), $CoCl_2 \cdot 6\ H_2O$ (200 mg), $CuSO_4 \cdot 5\ H_2O$ (10 mg), $NiCl_2 \cdot 6\ H_2O$ (20 mg), and $NaMoO_4 \cdot 2\ H_2O$ (30 mg). Cornsteep medium was made of (per L) 20 g of Cornsteep and 10 g of glucose (pH was adjusted to 7 with sterile NaOH-solution after sterilizing). Potato-dextrose-broth (PDB) was obtained from Difco. All other chemicals were obtained from Merck (p.a. quality). Media were sterilized at 121 °C for 30 min. In order to avoid precipitates, C and N-sources were sterilized separately.

Analytical methods. Concentrations of protected carboxylic acids and the protected ketone as well as their metabolites were measured by gas chromatography *(20,21)*. Cell dry weight (CDW) was determined by lyophilization *(21)*.

Biotransformation procedure. Biotransformations were performed in shake flasks (1 L) with four baffles containing 250 mL medium at 30 °C and an agitation of 130 rpm. Applied substrate concentrations are summarized in Table 2. *B. bassiana* was grown for 40 h in Cornsteep medium before the addition of the substrate and Witconate. *B. megaterium* was cultivated in glucose-min-

Table 1. Substrates used with [a]*B. megaterium*, [b]*B. bassiana*, [c]*C. lunata*, [d]*C. blakesleeana*, their unprotected equivalents and products obtained. Drawn are the following enantiomers: [1c](1*R*,2*R*); [2b](1*S*,3*S*); [2c](*R*); [3b](5*R*,7*R*)

Unprotected Substrate	HOOC-	HOOC-	O
Substrate	**1a** [a]	**2a** [acd]	**3a** [b]
Products	**1b** [a] **1c** [a]	**2b** [acd] **2c** [cd] **2d** [acd]	**3b** [b]

Table 2. Additive and substrate concentrations employed with *B. megaterium*, *B. bassiana*, *C. lunata*, and *C. blakesleeana*

	B. megaterium	*B. bassiana*	*C. lunata*	*C. blakesleeana*
γ(β-Cyclodextrin) / g L^{-1}	[a] 0.5, 1,0, 2.0, 3.0	n. t.	[b] 0.5, 1.0, 3.0	[c] 0.5, 1.0, 2.0, 3.0
Substrate conc. / mg L^{-1}	**1a**: 280		**2a**: 310	**2a**: 230
γ(Witconate) / g L^{-1}	0.5, 1.0, 2.0, 3.0	0.5, 1.0, 2.0, 3.0	n. t.	0.2, 0.6, 1.0
Substrate conc. / mg L^{-1}	1a: 320	**3a**: 400		**2a**: 300
γ(Triton X-100) / CMC	0.5, 1.5	n. t.	0.5, 1.5	0.5, 1.5
Substrate conc. / mg L^{-1}	**2a**: 300		**2a**: 300	**2a**: 300
γ(Tween 80) / g L^{-1}	0.5, 1.0, 2.0	n. t.	0.5, 1.0, 2.0	0.6, 1.0, 2.0, 3.0
Substrate conc. / mg L^{-1}	2a: 300		**2a**: 300	**2a**: 300
γ(Sodium tetraborate) / g L^{-1}	0.012, 0.120	n. t.	0.012, 0.120	0.012, 0.120, [d] 0.060
Substrate conc. / mg L^{-1}	2a: 300		2a: 300	**2a**: 300

[a] For each flask β-CD was dissolved in 10 mL of sterile water and added to the culture together with the ethanolic substrate solution.
[b] β-CD was added as a solid simultaneously with the ethanolic substrate solution.
[c] The ethanolic substrate solution was mixed with the sterile β-CD solution, prepared by dissolving β-CD in 15 mL H_2O
[d] 0.060 g L^{-1} of sodium tetraborate was added to one culture as a solid simultaneously with the saturated ethanolic substrate solution.
n.t. not tested

eral salt medium. After 10 h of growth, (CDW = 4.5 g L^{-1}), the cells were induced with 50 mg L^{-1} of the substrate. 5 h after substrate induction (CDW = 6.3 g L^{-1}) the substrate and the additives were added. *C. lunata* was incubated in modified medium E4 for 34 h, then the substrate and additives were added. *C. blakesleeana* forms pellets in submerged culture, differing in size and number under different conditions. In order to standardise biomass for the comparison of kinetic data, the fungus was grown in an 11-L bioreactor (medium E) as described by Kreiner *et al. (21)*. After 47 hours of growth the culture was distributed into 18 flasks in a sterile manner. Immediately afterwards the substrate and detergents or sodium tetraborate were added. The effects of β-CD were studied in further experiments. Czapek-Dox medium, which allowed the formation of smaller pellets, was inoculated with spores as described previously *(21)*. After 48 h of growth, the substrate and β-CD were added.

Additives and their application. β-CD (kleptose) was a gift from Roquette, Lestrem, France. Witconate SK, a mixture of linear sodium alkylbenzylsulfonates, was kindly donated by the Witco Corporation, Blue Island, USA. Tween 80 and $Na_2B_4O_7 \cdot 10\ H_2O$ were obtained from Merck, Germany and TritonX-100 from Serva, Heidelberg, Germany. These additives were used without further purification.

Concentrations of additives and substrates are summarized in Table 2. Unless otherwise stated substrates were added as saturated ethanolic solutions. β-CD was added either as a solid or dissolved together with the substrate solution as described in Table 2. Solid Witconate and Tween 80 were added simultaneously with the substrate solution. The substrate was solubilized either in 3.4 mL or 9.8 mL of Triton X-100 (0.5%). These solutions were then added to the cultures to give a detergent concentration of about 0.5 and 1.5 fold CMC in the culture broth. 0.03 g and 0.30 g of $Na_2B_4O_7 \cdot 10\ H_2O$ were dissolved in 2 mL and 5 mL of water at 60 °C, respectively. Ethanol was added to each solution to yield a final volume of 10 mL. 1 mL of each of these solutions was mixed with the respective substrate and then added to the shake flask culture.

Substrates and products. The synthesis of 2-cycloalkylbenzoxazoles and spirooxazolidines as well as substrate specificities and stereoselectivities of the hydroxylations of various benzoxazoles with *C. blakesleeana* and *B. megaterium* as well as of spirooxazolidines with *B. bassiana* are described by de Raadt *et al. (19,20)*. Table 1 presents a survey of the substrates used and the products obtained. Products formed by *C. lunata* were identified by GC with reference products from the transformation of 2-cyclopentylbenzoxazole with *C. blakesleeana*.

Results

Effects of different additives on the biohydroxylation reactions are presented in Figs. 1–5 showing the time-courses of the formation of the main hydroxylated products: *trans*-4(benzoxazol-2yl)cyclohexan-1-ol (**1b**) (*B. megaterium*), *trans*-3-(benzoxazol-2yl)cyclopentan-1-ol (**2b**) (*B. megaterium*, *C. lunata*, *C. blakesleeana*) and 4-*N*-benzoyl-1-oxa-4-aza-spiro[4.4]-nonan-7-ol (**3b**) (*B. bassiana*).

Effects of β-*Cyclodextrin*

B. megaterium. The effects of β-CD on the hydroxylation were found to be strongly dependent on the concentration of the additive (Fig. 1A). In the presence of 2 g L^{-1} and 3 g L^{-1} β-CD the yield of main product *trans*-4(benzoxazol-2-yl)cyclohexan-1-ol (**1b**) was reduced by 16% and 65%, respectively. The lower β-CD (0.5 g L^{-1}) concentration, however, was found to be slightly beneficial for the transformation which can be seen in the yield increase of 5%.

C. lunata. β-CD had a strongly positive effect on the formation of the main product during the transformation of 2-cyclopentylbenzoxazole (**2a**) with *C. lunata* (Fig. 1B). 0.5 g L^{-1} increased the rate of hydroxylation, resulting in a maximum concentration of the main product **2b** which exceeded the one found without β-CD by 65% at the end of the experiment. Positive results were also found with a concentration of 1.0 g L^{-1}, which led to an increase of the yield by 50%. In addition to the main product (Fig. 1B), the low β-CD concentrations (0.5 and 1.0 g L^{-1}) also increased the production rates as well as

the yields of the by-products. For example, the presence of 0.5 g L^{-1} β-CD led to an increase of the yield of the second alcohol **2d** up to 65%. With increasing amounts of β-CD the positive effect declined. 3.0 g L^{-1} β-CD showed no significant influence on the formation of the main product **2b**. However, the oxidation of this compound to the corresponding ketone **2c** was positively influenced (30% increase of yield).

C. blakesleeana. Kinetic data of main product *trans*-3-(benzoxazol-2yl)cyclopentan-1-ol (**2b**) formation during the biotransformation of 2-cyclopentylbenzoxazole (**2a**) in the presence of β-CD with *C. blakesleeana* are presented in Fig. 1C. In the presence of 0.5 g L^{-1} β-CD the production rate of the main product **2b** increased from 18 mg L^{-1} h^{-1} (control) to 34 mg L^{-1} h^{-1} within the first 3 h after substrate addition. As a consequence, the maximum concentration of **2b** was reached after 40 h, which was 34 h earlier as compared with the control culture. In addition, the oxidation to the corresponding ketone **2c** was accelerated in the presence of 0.5 g L^{-1} β-CD. With increasing β-CD concentrations, the positive effect on product **2b** production declined. The formation of the main product **2b** in the presence of 1.0 g L^{-1} β-CD was identical to the control with the exception that the maximum concentration of this product was increased. 2.0 and 3.0 g L^{-1} of β-CD reduced the production rate as well as the yields compared with the control.

Effects of Witconate

B. bassiana. The biohydroxylation of compound **3a** in the presence of 0.5 g L^{-1} Witconate tripled the rate of product formation within the first 3 h after substrate addition. Afterwards the rate declined, but was still twice as much as the control rate (Fig. 2A). As a result, the concentration of the hydroxylated product **3b** was increased by 95% 9 h after substrate addition. After this time the rates of product formation became identical for the transformation with and without Witconate. Finally, a 33% increase of the maximum product concentration was obtained in the presence of 0.5 g L^{-1} Witconate. The subsequent decline in the product concentration was due to the cleavage of the protecting group at low pH-values initiated by the excretion of acids by the fungus. Kinetic data for product formation in the presence of 1 g L^{-1} of Witconate were almost identical to those with 0.5 g L^{-1}, whereas the positive effect of Witconate declined with increasing detergent concentrations: 2 g L^{-1} Witconate still led to an increase of yield, but with a delay in product formation as compared with the low Witconate concentrations. Finally, in the presence of 3.0 g L^{-1} Witconate hydroxylation started after a lag-phase of 25 h.

B. megaterium. In contrary to the eucaryotic microorganism *B. bassiana*, Witconate had a strongly inhibitory effect on the biohydroxylation of **1a** with *B. megaterium*. In the presence of any Witconate concentration tested the substrate concentration remained constant during the experiment and hydroxylation of 2-cyclohexylbenzoxazole (**1a**) was not observed.

C. blakesleeana. Low Witconate concentrations were chosen for the hydroxylation of protected cyclopentanecarboxylic acid with *C. blakesleeana*, owing to the fact that these were found to be optimal for *B. bassiana*. However,

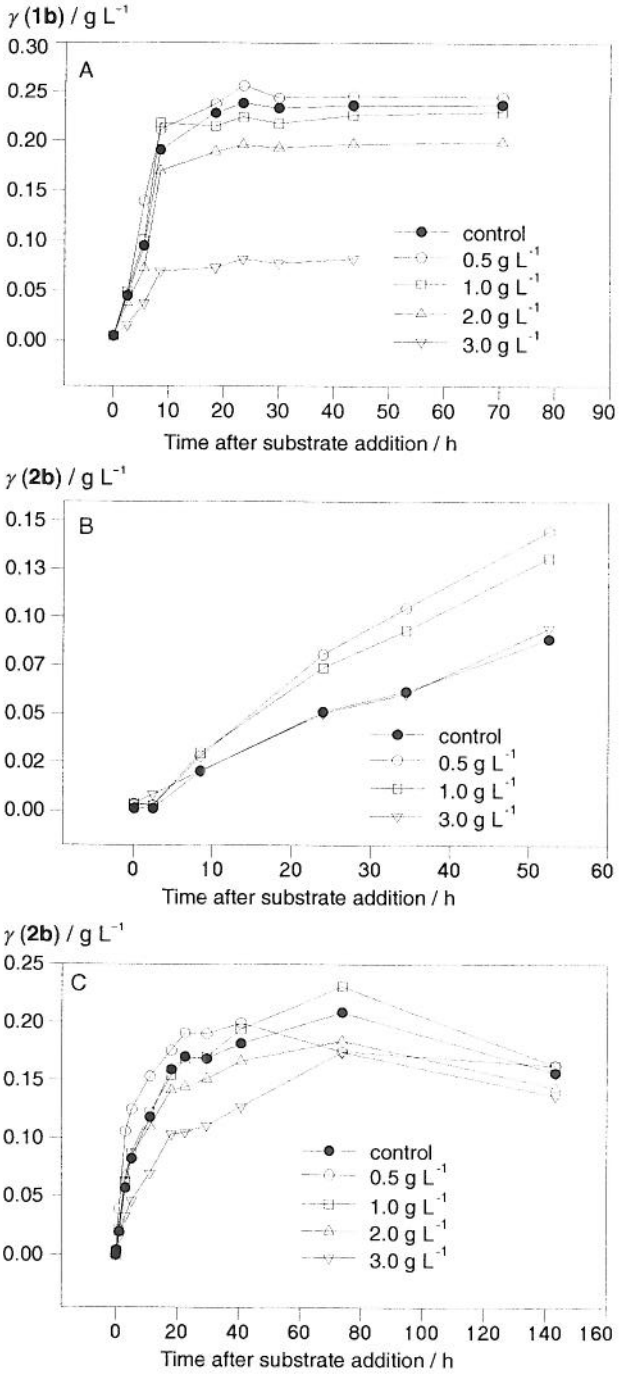

Fig. 1. Biohydroxylations in the presence of β-cyclodextrin (0.5–3.0 g L^{-1}). A. Biohydroxylation of **1a** with *B. megaterium* yielding **1b**. B. Biohydroxylation of **2a** with *C. lunata* yielding **2b**. C. Biohydroxylation of **2a** with *C. blakesleeana* yielding **2b**.

the effects were not as positive as expected (Fig. 2B). Only 0.2 g L^{-1} Witconate resulted in a slight increase of maximum product concentration. However higher Witconate concentrations decreased both hydroxylation and further oxidation.

Effects of Triton X-100

B. megaterium. Triton X-100 was found to exert an inhibitory effect on the biohydroxylation of 2-cyclopen-

tylbenzoxazole (**2a**) with *B. megaterium*. Compared with the control, the lower Triton X-100 concentration (0.5 × CMC) tested resulted in a 62% decrease of the maximum concentration of the main product **2b**. In the presence of 1.5 × CMC Triton X-100 only 22% of the product concentration, compared with the control, was obtained.

C. lunata. As can be seen from Fig. 3A, Triton X-100 finally led to a slight increase (15%) of maximum product concentration. The rate of hydroxylation, however, was reduced by the higher Triton X-100 concentrations (1.5 × CMC), but hydroxylation continued longer than with the control.

C. blakesleeana. The biotransformation of 2-cyclopentylbenzoxazole (**2a**) with *C. blakesleeana* was accelerated in the presence of 0.5 × CMC of Triton X-100 (Fig. 3B). The maximum concentration of the main product **2b** was achieved 19 h after substrate addition, which was 12 h earlier as with the control. In addition, the rate of oxidation to the corresponding ketone **2c** was increased. In the presence of 1.5 × CMC Triton X-100, an increase of 13% of the maximum concentration of the main product **2b** and of 23% of the corresponding ketone **2c** was found.

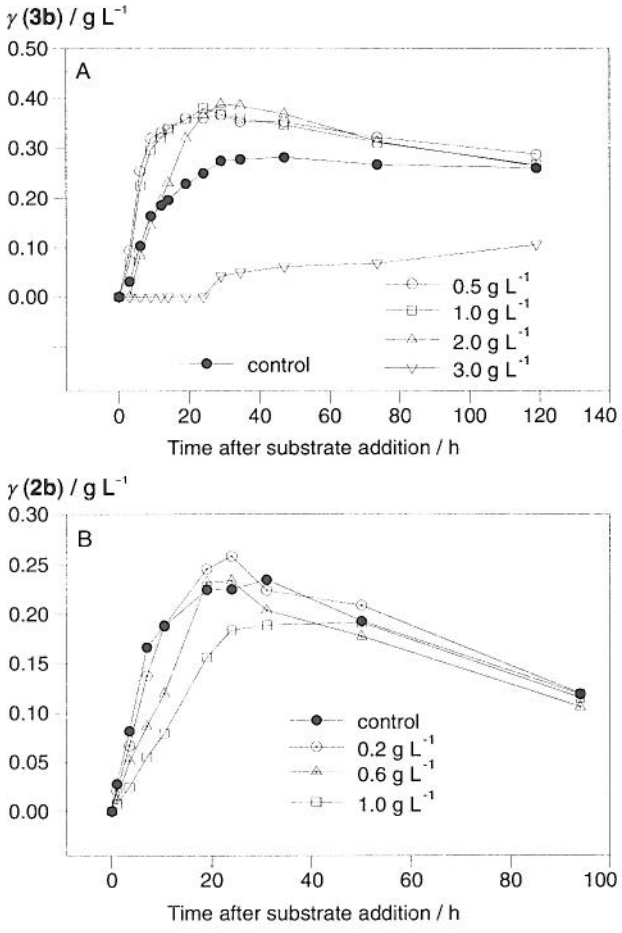

Fig. 2. Biohydroxylations in the presence of Witconate SK (0.2–3.0 g L^{-1}). A. Biohydroxylation of **3a** with *B. bassiana* yielding **3b**. B. Biohydroxylation of **2a** with *C. blakesleeana* yielding **2b**.

Effects of Tween 80

B. megaterium. In the presence of Tween 80 an inhibition of the hydroxylation was observed. Regardless of the detergent concentration (0.5, 1.0 and 2.0 g L^{-1}), a 55% decrease in yield of the main product, *trans*-3-(benzoxazol-2-yl)cyclopentan-1-ol (**2b**), was found. The formation of the by-product 2-(benzoxazol-2-yl)cyclopentan-1-ol (**2d**) was affected similarly.

C. lunata. Tween 80 was found to promote the hydroxylation of 2-cyclopentylbenzoxazole (**2a**) with *C. lunata*. All concentrations tested (0.5, 1.0 and 2.0 g L^{-1}) increased the maximum amount of the main product **2b**

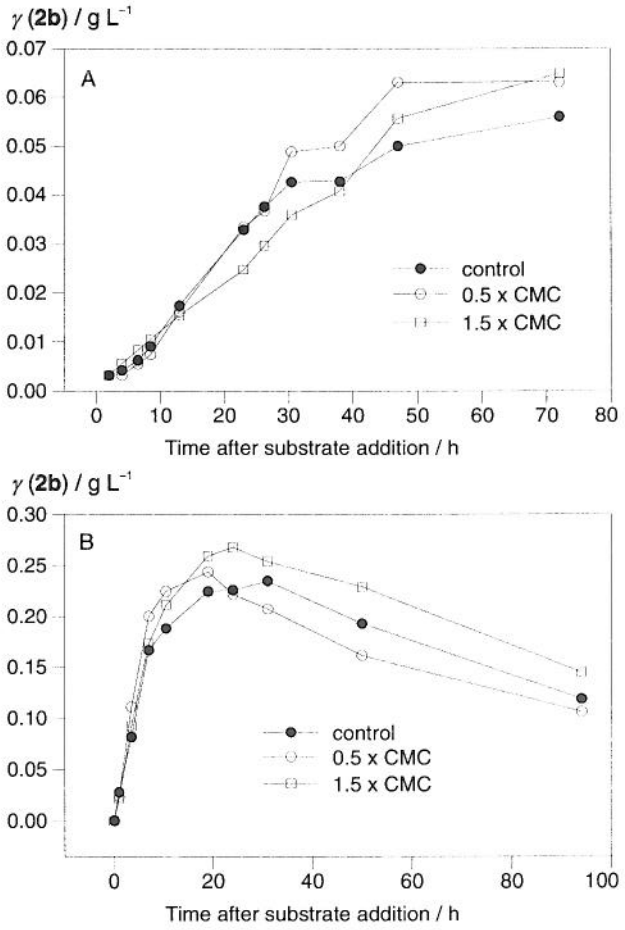

Fig. 3. Biohydroxylations in the presence of Triton X-100 (0.5 and 1.5 × CMC). A. Biohydroxylation of **2a** with *C. lunata* yielding **2b**. B. Biohydroxylation of **2a** with *C. blakesleeana* yielding **2b**.

(Fig. 4A). In particular, the presence of 1.0 g L^{-1}, which allowed a 65% increase of yield of this product, was found to be the most favouring. Similar effects were found for the formation of the by-product **2d**. Concerning the amount of Tween 80, concentrations exceeding 2.0 g L^{-1} can be assumed to hamper the biotransformation as this concentration had a reduced positive effect on the formation of the main product **2b** and no positive effect on the production of the by-product **2d**.

C. blakesleeana. The presence of Tween 80 had a slight positive influence on the biotransformation of

2-cyclopentylbenzoxazole (**2a**) with *C. blakesleeana* with respect to maximum product concentrations (Fig. 4B). Especially 2.0 g L^{-1} of Tween 80 were found to be beneficial, allowing a 14% increase of maximum concentration of *trans*-3-(benzoxazol-2yl)cyclopentan-1-ol (**2b**) (Fig. 4B) and a 12% increase of the concentration of the corresponding ketone **2c**.

Effects of Sodium Tetraborate

B. megaterium. The presence of sodium tetraborate (120 mg L^{-1}) had no influence on the biotransformation of 2-cyclopentylbenzoxazol (**2a**) with this bacterium.

C. lunata. The addition of sodium tetraborate decreased the production rate of the main product **2b** within the first 30 h after substrate addition, but resulted in a 14% increase in final yield. Similar effects were observed for the by-product **2d**. The further oxidation of **2b** to the ketone **2c** was not significantly influenced by sodium tetraborate.

C. blakesleeana. The influence of sodium tetraborate on the biotransformation with *C. blakesleeana* was found to be concentration dependent. (Fig. 5). Whereas the maximum concentration of the main product **2b** was reduced by 21% in the presence of 120 mg L^{-1} sodium tetraborate, the lower additive concentration of 12 mg L^{-1} allowed a 25% increase of the maximum yield of this product. Similar effects were found for the further oxidation to the ketone **2c**. Contrary to the previous method involving mixing of the substrate with the sodium tetraborate solution prior to addition, the direct addition of solid sodium tetraborate (60 mg L^{-1}), simultaneously with the substrate solution, had no influence on this biotransformation.

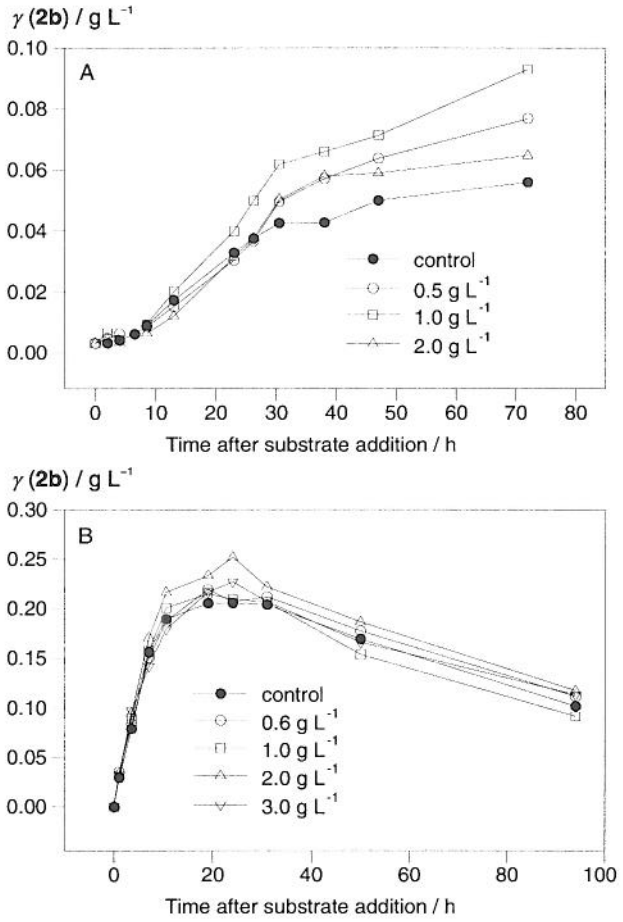

Fig. 4. Biohydroxylations in the presence of Tween 80 (0.5–3.0 g L^{-1}). A. Biohydroxylation of **2a** with *C. lunata* yielding **2b**. B. Biohydroxylation of **2a** with *C. blakesleeana* yielding **2b**.

Discussion

β-CD was found to be beneficial for biohydroxylation reactions, regardless of the type of microorganism. A molar ratio of 4 to 2 moles of substrate per mole of β-CD (0.5 and 1.0 g L^{-1}) appeared to be optimal. Inhibitory effects, however, emerged with increasing β-CD concentration (about 2 g L^{-1}). β-CDs can interact not only with the substrate/product molecules, but also with the respective microorganism *(11–13)*. Especially cell membranes are thought to be susceptible to these compounds *(13)*. Phospholipids can form complexes with CDs and therefore CDs can extract membrane components and consequently disturb the integrity of a membrane. Nevertheless, natural CDs are generally regarded as being nontoxic *(13)*.

Johnson *et al. (23)* found that Ultrawet DS-30, which is equivalent to Witconate SK, exerts a positive influence on the biohydroxylation of N-acylcyclohexylamines with *B. bassiana*. The results presented in this paper, where spirooxazolidines were used as substrates, support this observation. However, such significant positive results could not be obtained with the other microorganisms considered here.

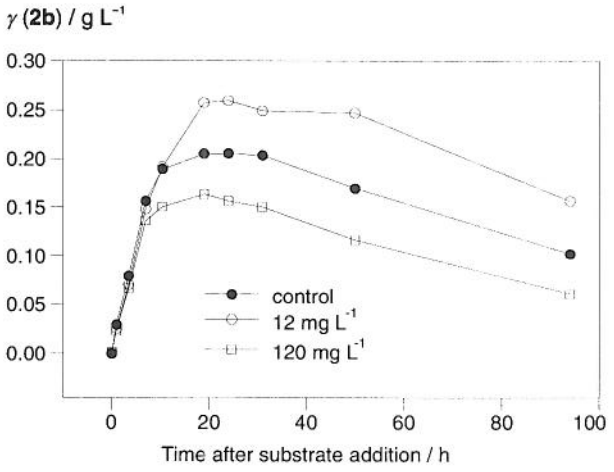

Fig. 5. Biohydroxylation of **2a** with *C. blakesleeana* in the presence of sodium tetraborate (12 and 120 mg L^{-1}) yielding **2b**.

Detergents were found to exert a positive influence on the hydroxylation reactions usually when applied in low concentrations. It is well known that surfactants can be toxic to bacteria because they integrate in the cell membrane which results in an alteration of membrane permeability *(22)*. For this reason detergents such as Triton X-100 have been used as means of controlled permeabilization of cells, allowing the free diffusion of low molecular mass molecules across the cell membrane,

Table 3. Effects of additives on biohydroxylation reactions with various procaryotic and eucaryotic microorganisms

	B. megaterium	*B. bassiana*	*C. lunata*	*C. blakesleeana*
β-Cyclodextrin	+	n. t.	+	+
Witconate	– –	+	n. t.	–
Triton X-100	– –	n. t.	+	+
Tween 80	– –	n. t.	+	+
Sodium tetraborate	– –	n. t.	+	+

+ slightly positive , ++ very positive, – negative,
– – inhibition of the biohydroxylation in comparison to the transformation without additive,
n. t. not tested

consequently increasing biocatalytic activity *(22)*. The effects of detergents on the cells are generally dependent on the applied concentration and on the chemical composition of the cells.

Concerning the effects of investigated surfactants, there are differences between procaryots and eucaryots (Table 3). The bacterium *B. megaterium* seemed to be more sensitive towards the applied surfactants than the fungi tested. In the presence of the non-ionic detergents Tween 80 and Triton X-100, the maximum concentration of the main product was reduced by 55% and 62%, respectively. In the presence of Witconate hydroxylation was not observed. The finding that *B. megaterium* shows a higher sensitivity towards the ionic detergent Witconate than to the non-ionic Tween 80 and Triton X-100 is in accordance with the general fact that non-ionic surfactants are regarded as being less toxic for bacteria than ionic surfactants *(17)*.

The presence of sodium tetraborate (12 mg L^{-1}) resulted in a 25% increase of the maximum concentration of the main product **2b** during the biohydroxylation of 2-cyclopentylbenzoxazole (**2a**) with *C. blakesleeana*. These positive findings may be due to changes in the permeability of the cells to the substrate as was argued by Lee *et al. (24)* for the improvement of the transformation of 16-α-hydroxycortexolone-16,17-acetonide with a mixed culture of *Arthrobacter simplex* and *Curvularia lunata* employing the same additive. However, with our substrates, the hydroxylation with *C. lunata* was not affected as positively by this additive as with *C. blakesleeana*.

Table 3 summarises the effects of β-CD, three detergents, and sodium tetraborate on the biohydroxylation reactions under consideration in this paper. The detergents tested were an anionic benzylsulfonate detergent (Witconate SK), a sorbitan ethoxylate surfactant (monooleate Tween 80), and an alkylphenol ethoxylate (Triton X-100). The data presented here suggest that use of these additives to enhance biohydroxylation reactions has to be investigated for each microorganism and optimal conditions have to be established. Results of studies on the effects of surfactants in biodegradation experiments are also diverse *(25)*. Clearly, the effect of an additive on microbial reactions depends on the system employed, that is on various factors such as the concentration of the additive and the substrate/product as well as the interaction between these compounds *(11,17)* and the microbial cells *(13,22)*. Regarding this work it can be expected, however, that appropriate modulators can be found for fungal systems more easily than for gram-positive bacteria, as the former seem to be less sensitive towards the possible inhibitory impacts of additives. In this respect, all the additives, except the ionic detergent Witconate, were beneficial for all biohydroxylations investigated with *C. lunata* and *C. blakesleeana*, whereas only β-CD favoured reactions with *B. megaterium*.

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Utjecaj aditiva na biohidroksilaciju zaštićenih karboksilnih kiselina i ketona

Sažetak

Istraživan je utjecaj β-ciklodekstrina (β-CD), anionskog detergenta Witconate SK, dvaju neionskih površinsko-aktivnih spojeva (Triton X-100 i Tween 80) te natrijeva tetraborata na biohidroksilaciju zaštićenih cikličkih karboksilnih kiselina i ketona s pomoću Bacillus megaterium i gljive Beauveria bassiana, Curvularia lunata i Cunninghamella blakesleeana u pokusima na tresilici.

Bakterija B. megaterium puno je osjetljivija na spomenute površinsko-aktivne spojeve od ispitivanih gljiva. Prisutnost Tritona X-100 (0,5 puta više od kritične koncentracije micelija CMC) i Tweena 80 (0,5-2,0 g L^{-1}) utjecala je na sniženje maksimalne koncentracije glavnog produkta hidroksilacije za 62, odnosno 55%. Primjenom detergenta Witconate nije uopće došlo do hidroksilacije. Samo se β-CD (0,5 g L^{-1}) u prisutnosti B. megaterium, pokazao nešto učinkovitijim pri transformaciji, a ukupno je iskorištenje bilo za 5% veće.

Suprotno rezultatima dobivenim s B. megaterium navedene gljive u prisutnosti svih ispitivanih aditiva dale su veći postotak biotransformacije. Općenito najbolji su rezultati dobiveni s malim koncentracijama detergenata (do 1 g L^{-1}). Najpovoljniji rezultati za transformaciju s pomoću C. lunata postignuti su s 0,5 g L^{-1} β-CD i 1 g L^{-1} Tween 80, čime se maksimalno iskorištenje glavnog produkta hidroksilacije povećalo za 65%. Dodatak od 12 mg L^{-1} natrijeva tetraborata povećao je maksimalnu koncentraciju glavnog produkta u prisutnosti C. blakesleeana. Primjenom B. bassiana uz detergent Witconate (0,5 i 1,0 g L^{-1}) postignuto je 33%-tno povećanje maksimalne koncentracije produkta.