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Biocatalytic asymmetric hydroxylation of hydrocarbons by free and immobilized *Bacillus megaterium* cells

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Abstract

Screening of soil bacteria with allylbenzene resulted in a *Bacillus megaterium* strain, which hydroxylates simple hydrocarbons in high enantiomeric excess (ee up to 99%). Benzylic and nonbenzylic hydroxylation products were obtained, without the usually observed high preference for the benzylic position. The immobilization of the *B. megaterium* cells in alginate gel effectively improved the stability of the cells and increased the amounts of products formed, without loss of enantioselectivity. The product ratio (α vs. β hydroxylation) was shifted towards benzylic hydroxylation, which suggests that at least two hydroxylating enzymes with distinct regioselectivity are involved. Comparison to free-cell fermentations in small- and large-scale bioreactors (up to 2000 ml) showed that the use of immobilized cells is advantageous, as they are easier to handle and yield higher amounts of oxidation products.

Keywords: *Bacillus megaterium*; Hydroxylation; Hydrocarbons; Immobilization; Alginate

1. Introduction

Asymmetric hydroxylations provide a powerful method to obtain potentially useful, optically active, oxyfunctionalized synthons of unactivated hydrocarbons. There exist only a few classical synthetic methods that may be employed for this purpose [1,2], and, recently, a variety of metal catalysts have been developed for the stereoselective hydroxylation of unfunctionalized hydrocarbons [3,4]. Nevertheless, the enantioselectivity, as well as the substrate range, of many of such reactions still require substantial improvement.

Alternatively, microorganisms have already been successfully applied for the selective oxygenation of organic substances, especially the unactivated sites in hydrocarbons, for which the mild conditions and efficacy of bioreactions remain unchallenged [5]. Along these lines, the majority of the current work focuses on the hydroxylation of steroids, terpenes or other complex natural products [6]. For these biotransformations mostly fungi such as *Rhizopus nigricans*, *Mortiella isabellina* and *Cunninghamella elegans* have been employed [7–9], and high regio- and enantioselectivities have been reported [10–14]. In contrast, the hydroxylation of xenobiotics is far more difficult because, unlike natural products, these com-

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pounds are not readily introduced into the cell for metabolism. To improve the yields of such biotransformations, immobilization in alginate gel ("gel entrapment") presents a widely used technique, since it is straightforward to apply to viable cells and purified enzymes [15]. Such cell immobilization has been used since the 1950s and has nowadays been exploited for industrial applications in the agricultural and pharmaceutical fields [16]. Of the numerous advantages, the more important ones are the easy separation of the cells, their reusability, the sharp reduction of metabolic by-products, and the improved stability of the cells [17,18].

In this contribution, we present our results for the hydroxylation of simple hydrocarbons by a *Bacillus megaterium* strain, which was obtained from topsoil by a selective screening procedure. The biotransformations reported herein afford the benzylic as well as the nonbenzylic (usually not favored according to literature [19]) alcohols in high enantiomeric excesses and with little, if any, oxidation by-products (frequently described for other biotransformations [5]). Furthermore, it was demonstrated that immobilization of this bacterial strain in alginate gel increases the yields of the hydroxylation products with preservation of the high enantioselectivity through the significantly improved stability of the cells.

2. Experimental section

2.1. General

Plate-Count Agar was purchased from Difco (Difco Laboratories, Detroit, MI, USA). AmpliTaq Gold®-DNA-polymerase was obtained from Perkin Elmer (Perkin Elmer, Weiterstadt, Germany). 3-Phenylpropen-3-ol (**2b**) was prepared by reduction of 3-phenylpropin-3-ol with $LiAlH_4$ as described in literature [20]. 1-Phenylpentan-3-ol (**2eγ**) and 5-phenylpentan-2-ol (**2eδ**) were prepared by Grignard reactions of the respective aldehydes under standard conditions [21]. All other compounds were acquired from Aldrich, Sigma or Fluka (Sigma-Aldrich Chemie, Steinheim, Germany). A Wolf SanoClav-MCN autoclave was used for sterilization (Wolf, Geisslingen, Germany). A laminar AirFlow bench was used for sterile work (NuAire, Plymouth, MN, USA). Beckman J2-21 centrifuge was applied for cell separation (Beckman, Fullerton, CA, USA). Multidimensional gas chromatography was carried out with a Sichromat 2 (Siemens, München, Germany). 373A automatic sequencer was used for the RNA determinations (ABI division of Perkin Elmer, Weiterstadt, Germany).

2.2. Protocol for the bacterial screening of topsoil

Plates with Plate-Count Agar or minimal medium agar (Dworkin and Foster [22]; glucose 0–5 g/l final concentration) and various amounts of allylbenzene (5–25 μl per plate) were inoculated with soil bacteria in aqueous media. For this purpose, 1 g of soil was stirred with 50 ml of demineralized water for approximately 30 min and 100 μl were transferred to each plate. Plates with the same amount of allylbenzene were inoculated at different dilutions (10- to 1000-fold), which were prepared from the initial broth. Other plates, which contained allylbenzene as the sole carbon source, were inoculated by transferring bacteria from the plates of soil bacteria grown without substrate addition, which facilitated to observe visually the growth of the transferred bacteria. All plates were incubated at 30°C for 1 week and checked twice per day; cultures with outstanding growth were transferred to another plate without allylbenzene to ensure that only a single strain was isolated. No strain was able to grow on plates whose medium did not contain glucose. Finally, each isolated strain was grown in a liquid culture in the presence of a hydrocarbon as described in the Biotransformation section in order to check if new products had been formed.

2.3. General procedure for the biotransformation of the hydrocarbon

Liquid minimal media (75 ml) were prepared according to Dworkin and Foster [22], 5 ml/l of a mineral salt solution [23] was added and autoclaved (121°C, 16 min). The culture was maintained under sterile conditions during the addition of glucose (5 g/l). For inoculation of liquid media, cultures were taken from freshly grown plates. Under sterile condi-

tions, c. 100 μmol of the hydrocarbon substrate was added and the liquid culture was allowed to grow for c. 18 h at 30°C. Control experiments without bacteria were carried out to verify the stability and authenticity of the starting material; no oxidation products could be observed under these conditions. After c. 17 h, the culture was worked up by 15-min sonication of the bacterial broth and centrifugation at $15\,000 \times g$ for 20 min. The supernatant solution was extracted with 120 ml pentane/dichloromethane (2:1) by liquid–liquid extraction for 24 h. The extract was dried over Na_2SO_4 and distilled over a Vigreux column (40°C, 990 mbar) to prevent loss of volatile compounds by evaporation. The products were submitted to gas-chromatographic analysis, coupled with mass spectrometry, and their identity was verified by comparison with authentic reference samples. The enantiomeric excess of the alcohols was determined by multi-dimensional gas chromatography (MDGC).

Assignments of the absolute configurations were made with the circular-dichroism–exciton-chirality method [24] (details to be published separately) and, wherever possible, confirmed by comparison with authentic enantiomerically pure reference samples.

2.4. Immobilization of B. megaterium cells and biotransformation of hydrocarbons by immobilized cells

A liquid culture (75 ml) was grown to mid-log phase and centrifuged mildly (c. $3000 \times g$). The bacterial pellet was resuspended in 2 ml of an isotonic NaCl/$MgSO_4$ solution and 3 ml of a Na-alginate (3% w/v) solution was added. After mixing, the cell suspension was added dropwise to a new medium of 0.1 M $CaCl_2$, 5 g/l glucose and 5 ml/l of a trace-element solution [23]. The gel beads were allowed to harden for 1 h without agitation and after an additional 30 min at 30°C and 120 rpm, the substrate was added. Work-up conditions were as described in the Biotransformation section except that the beads were collected by filtration and immediately resuspended in fresh liquid medium, to initiate a new catalytic cycle.

2.5. Large-scale (2 l) fermentations

For larger-scale fermentations, a Bioflo 3000 bioreactor (New Brunswick, Edison, NJ, USA) was used. Two starter cultures of *B. megaterium* (75 ml), which were grown overnight, were added to the liquid medium (2000 ml) described in the Biotransformation section. During the mid-log phase of growth, which was monitored by the oxygen saturation of the medium, the substrate was injected through a sealed port. The inflow of air was cut off immediately and the agitation was increased from 250 to 350 rpm. This step was necessary since it was noted that the steady flow of air rapidly evaporated the volatile substrate from the bioreactor. After a 17-h incubation time, the bacterial broth was pumped off and centrifuged at $15\,000 \times g$. Faster cross-flow filtration was also attempted, which led to significantly reduced recovery of the products. The cell-free supernatant was liquid–liquid extracted and analyzed like described in the Biotransformation section.

2.6. Induction of the cytochrome P-450 activity

Except for the addition of the inducer, the conditions for all of the biotransformations remained as described above. The inducer phenobarbital [25] (4 mmol/l) was added during the mid-log phase, and 30 min later, addition of the substrate followed.

2.7. Scanning electron microscopy of gel beads

The alginate gel beads were prepared as described in the Immobilization section. The beads were separated from the immobilization medium by filtration and then resuspended in 6.25% buffered glutaraldehyde (0.1 M Tris–HCl buffer, pH 7.0 with 0.1 M $CaCl_2$). After 24 h at 4°C, the beads were washed with buffer, attached to coverslips, stepwise dehydrated in acetone, and critical-point dried with CO_2 (critical-point drier, BAL-TEC, Schalkmühle, Germany). The specimens were sputtered with 30-nm-sized gold particles and photographs were taken on a Zeiss DSM 942 scanning electron microscope (Zeiss, Oberkochen, Germany).

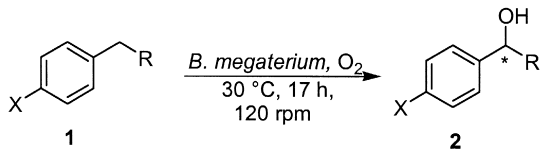
3. Results and discussion

An adequate screening procedure to select suitable soil bacteria for the biotransformation of ary-

lalkanes had to be developed. Allylbenzene was chosen as the selecting substrate because it possesses three different oxidation sites for its detoxification as a biologically harmful substance, namely the phenyl ring, the benzylic position, and the double bond. This screening procedure resulted in three different microbial species when various amounts (5–25 μl) of allylbenzene and glucose (0–10 g/l) were applied to soil samples of different origin. For the identification of the isolated bacteria, they were subjected to sequencing of the first 300 bp of their small subunit ribosomal RNA gene (*16s / 18s*) by means of the Taq-cycle-DyeDeoxy™-terminator technique [26–29]. The resulting nucleotide sequences (variable region V1–V3 of *16s*-like-rRNA) were compared to sequence data of the European Molecular Biology Laboratory (EMBL) database, which disclosed that an *Arthrobacter sp.*, a *Pseudomonas sp.* and a *Bacillus sp.* had been isolated. The *Bacillus* strain yielded the *R* enantiomer of the α-hydroxylation product (benzylic C–H insertion) of allylbenzene with an ee value of 70%, whereas the other strains were ineffective for oxidative biotransformations of this substrate. Therefore, the effective bacterium was more rigorously characterized and determined to be a *B. megaterium* strain.

To elucidate the scope of substrate acceptability by *B. megaterium*, several arylalkanes were used for the biotransformation (Scheme 1). *B. megaterium* was incubated with c. 100 μmol of the respective hydrocarbon for 17 h, the cells were removed by centrifugation, and liquid–liquid extraction of the growth medium was carried out for product isolation. Because the products and the substrates are quite volatile, the solvent was removed by distillation through a Vigreux column to prevent loss due to evaporation. Finally, the products were submitted to gas-chromatographic analysis and mass spectrometry. The enantiomeric excess was determined by multi-dimensional gas chromatography (MDGC) on cyclodextrin columns. The absolute configurations were established by comparison with authentic reference samples, wherever available, and by employing the circular-dichroism–exciton-chirality method [24] (details will be published separately).

Propylbenzene (**1a**), allylbenzene (**1b**), and (*p*-bromo)propylbenzene (**1c**) yielded solely the α-hydroxylation products in very similar enantiomeric



1 2

	R	X
a	C_2H_5	H
b	$CH{=}CH_2$	H
c	C_2H_5	Br
d	$CH(CH_3)_2$	H
e	$(CH_2)_3CH_3$	H

1f 

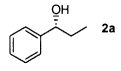
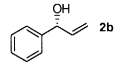
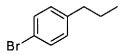
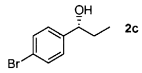
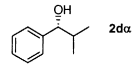
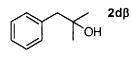
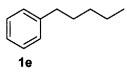
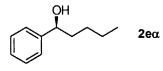
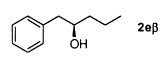
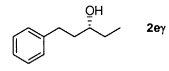
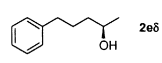
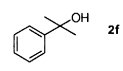
Scheme 1. Microbial asymmetric oxygen-atom insertion into C–H bonds of hydrocarbons with *B. megaterium*.

excesses of approximately 70%, in favor of the *R* enantiomers (Table 1, entries 1–3). The *p*-bromo derivative **1c** was converted almost quantitatively to the respective alcohol; this higher reactivity might be due to its increased lipophilic character in comparison to propylbenzene (**1a**). The better interaction of substrate **1c** with the highly lipophilic bacterial membrane increases its availability to the cell, which in turn facilitates its enzymatic conversion. The similar enantioselectivity of the microbial hydroxylation of all three substrates shows that neither the double bond in **1b** nor the *para*-bromo substituent in **1c** significantly perturb the binding of the substrate in the active site of the enzyme.

The introduction of a branched alkyl chain like in isobutylbenzene (**1d**) leads to the regiomeric α- and β-hydroxylation products (Table 1, entry 4). Especially interesting to note is that under standard reaction conditions the β product is formed preferentially, since usually microbial benzylic hydroxylations of arylalkanes are favored [7]. Furthermore, the **2dα** regioisomer is obtained in 91% enantiomeric excess in favor of the *R* enantiomer. Presumably, the increased steric demand of the isopropyl group is the reason for the significantly improved enantioselectivity in this biotransformation.

The tertiary CH position in (1-methyl)ethylbenzene (**1f**) is also almost quantitatively hydroxylated to yield the cumyl alcohol (**2f**). No other products were formed in the above biotransformations and control experiments without bacteria yielded no hydroxylated products with any of the substrates used. For pentylbenzene (**1e**), in addition to the C–H insertion products, oxidation of the respective alcohols resulted in small amounts of ketones (Table 1, entry 5; percentage given in parenthesis). Therefore, it may be concluded that oxidase activity is restricted to substrates with alkyl chains larger than four carbon atoms. In addition to α and β hydroxylation, the γ and δ regiomers were also obtained for the substrate **1e** in a 3:1 ratio (*S*)-1-phenyl-1-pentanol [(**2eα** + **2eβ**):(**2eγ** + **2eδ**)]. With regard to the enantioselectivity, it is striking that the biotransformation of pentylbenzene (**1e**) yielded the *S*-configured alcohols, (**2eα**) and (*S*)-5-phenyl-2-pentanol (**2eδ**), while for the **2eβ** and **2eγ** regiomers, the usual *R* enantiomers were preferentially formed. This distinct regiomer-dependent sense in the enantioselectivity cannot be explained in terms of subsequent kinetic resolution of the chiral alcohols **2e** through enantios-

Table 1
Regio- and enantioselectivities of the oxygen-atom insertion into the C–H bond of hydrocarbons by batch cultures of *B. megaterium*

entry	Substrate	convn[a] [%]	product	selectivity [%] regio[b]	selectivity [%] enantio[c]
1	 **1a**	63	 **2a**	100	74 (*R*)
2	 **1b**	49	 **2b**	96[d]	70 (*R*)
3	 **1c**	95	 **2c**	100	68 (*R*)
4	 **1d**	43	 **2dα**	31	91 (*R*)
			 **2dβ**	69	
5	 **1e**	89	 **2eα**	29 (4)	42 (*S*)
			 **2eβ**	33 (3)	88 (*R*)
			 **2eγ**	9 (3)	86 (*R*)
			 **2eδ**	14 (5)	> 99 (*S*)
6	 **1f**	95	 **2f**	100	-

[a] Conversion of hydrocarbons determined by GC analysis; error limit ± 2% of the stated values.
[b] Ratio of regioisomeric alcohols; in the case of ketone formation, the product distribution is normalized to 100%, and the amount of the corresponding ketone is given in parenthesis.
[c] Enantiomeric excesses (ee values) were determined by multidimensional gas chromatography with a cyclodextrin column.
[d] Small amounts (c. 4%) of cinnamyl alcohol were formed.

elective oxidation, as the amount of ketones found is too low to alter significantly the enantiomeric excess of the alcohols. Therefore, in the case of *S*-alcohol formation, either different enzymes are involved or the substrate is positioned differently in the enzyme cavity due to the longer alkyl chain and, thus, the sense of the enantioselectivity is reversed.

In an effort to increase the productivity of the biotransformation, the *B. megaterium* cells were immobilized in alginate gel ("gel entrapment") [15]. Gel beads were formed in 0.1 M $CaCl_2$ solution, which contained trace elements and glucose. After addition of the model substrate **1d**, the biotransformation was allowed to proceed for 17 h. Then, the beads were collected by filtration and immediately resuspended in fresh medium. Subsequently, new substrate was added again and another catalytic cycle was initiated. The filtrate of the previous cycle was extracted as described before and the products were analyzed by gas chromatography coupled with mass spectrometry and by multi-dimensional gas chromatography. Five catalytic cycles within 5 days were carried out, and it could be shown that during this period, the cells remained viable and performed the biotransformation of the model substrate **1d** (Fig. 1). It is especially significant that the enantiomeric excess remained at approximately 90% in favor of the *R* enantiomer for all catalytic cycles. Therefore, it may be concluded that the enantioselectivity does not depend on the amount of the product formed. Furthermore, the change in the growth conditions, which was necessary for the preparation of the immobilized cells, significantly affected the product distribution (Fig. 1). In contrast to free cell cultures, for the immobilized bacteria the α-hydroxylation product is formed preferentially. This shift in the product distribution indicates that the bacteria, despite their immobilization, are still able to express the hydroxylating enzymes, and thereby change the regioselectivity of the C–H insertion to correspond to the transcription of the monoxygenases. This implies that two or more hydroxylating enzymes with distinct regioselectivities are involved in these biotransformations. Certainly, the highest amounts of products were observed for the first catalytic cycle, whereas especially the second cycle displayed a sharp decrease of the amount of products formed in comparison to the first cycle (Fig. 1). The decrease between cycles II–IV is quite moderate, but, again, becomes more prevalent between cycles IV and V.

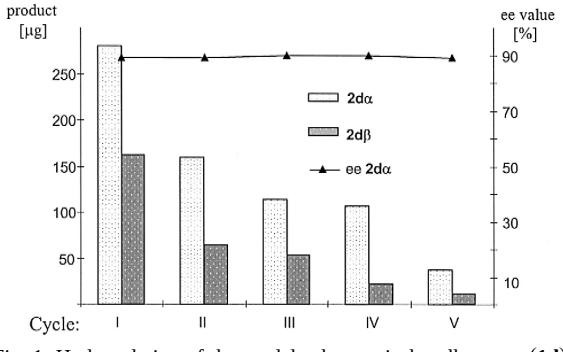


Fig. 1. Hydroxylation of the model substrate isobutylbenzene (**1d**) by immobilized *B. megaterium* cells; the bacteria were used for five cycles over a period of 5 days.

The diminished catalytic activity is due to cellular death which brings about a decline of the total amount of enzymes available for the biotransformation. Nevertheless, four cycles of the biotransformation may be carried out in quite high catalytic activity. This constitutes a sharp increase in the productivity compared to free cell cultures, especially since the latter usually die after completion of one cycle. The increased productivity becomes even more remarkable when the absolute amounts of products are directly compared to free cell cultures. A regular free-cell batch culture yields about half of the total amounts of products (**2dα** + **2dβ**) compared to the first catalytic cycle of a culture of immobilized bacteria (Fig. 2), while equal amounts of total products compared to free-cell cultures (Fig. 2) are obtained for cycle III (Fig. 1). This means that immobilized bacteria display a much higher catalytic activity for a longer period of time, because they do not undergo cellular growth as fast as free cells and, thus, are not as quickly deactivated. Furthermore, large-scale fermentations (2000 ml) in comparison to shaking-flask cultures (75 ml) show that the same product ratio was achieved with only a moderate increase in productivity per 100 ml of medium after optimization of the biotransformation conditions for the larger bioreactor (Fig. 2).

Addition of phenobarbital [25] as a potent inducer of cytochrome *P*-450 activity effectively induces the enzyme for the β hydroxylation, but it fails to improve the α hydroxylation. These results are con-

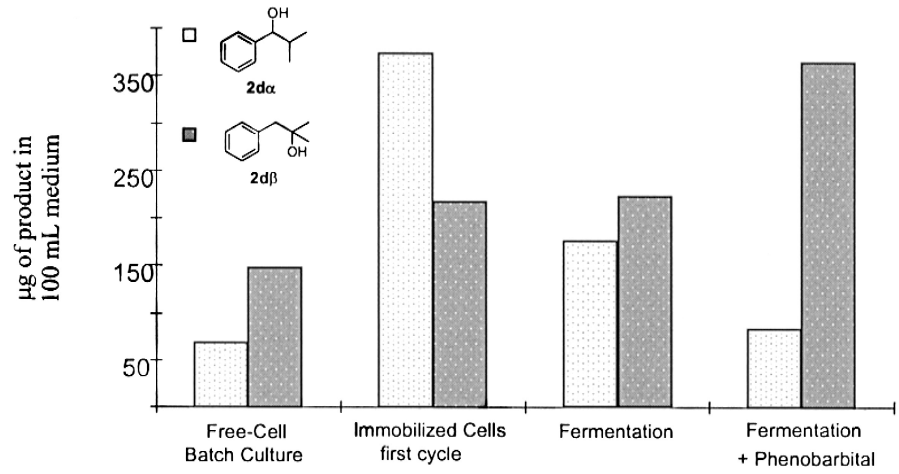


Fig. 2. Comparison of the C−H insertion of substrate **1d** by different preparations of *B. megaterium* cells. Free cells were used in batch cultures (shaking-flask) and in large-scale fermentations with and without the addition of phenobarbital (fermentation; fermentation + phenobarbital); harvesting the bacteria of a regular shaking-flask culture during mid-log phase resulted in comparable amounts of immobilized cells; products for the first catalytic cycle are shown (immobilized cells, first cycle).

sistent with data obtained from similar shaking-flask batch cultures grown in the presence of phenobarbital (data not shown). The shift of the regioselectivity in those cultures is once again brought about by a change in external growth conditions which clearly demonstrates that two or more monooxygenases with distinct regioselectivities appear to be involved in this biotransformation. If only one enzyme were to participate, the yields, but not the product distribution, should be altered. Thus, we conclude that immobilized bacteria yield the highest amount of products and may be handled more readily than larger-scale fermentations. The latter particularly cause problems during the product extraction, as they require large amounts of solvents and large-scale equipment to accommodate several liters of bacterial medium.

Finally, to gain insight into the structure of the immobilized biocatalyst, the alginate beads were subjected to scanning electron microscopy. The surface

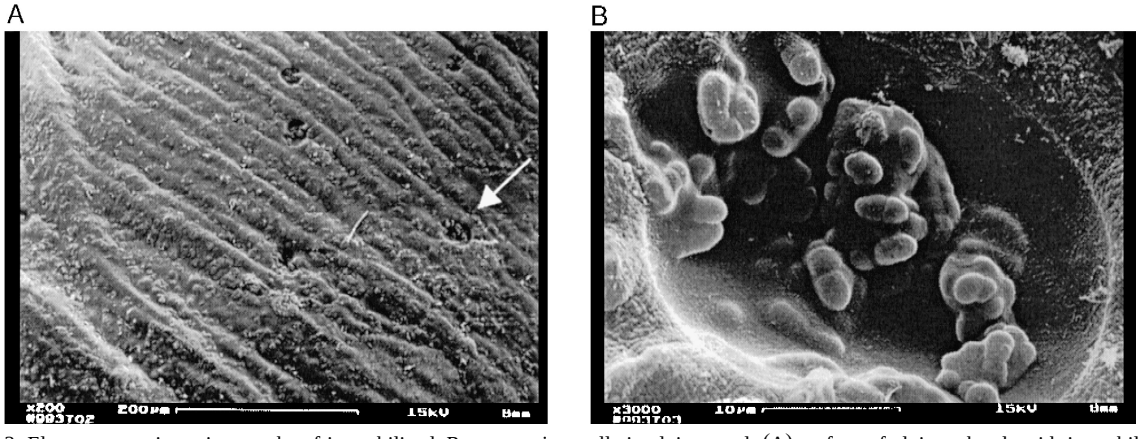


Fig. 3. Electron-scanning micrographs of immobilized *B. megaterium* cells in alginate gel; (A) surface of alginate beads with immobilized bacterial cells below the surface and in crater-type structures, indicated by the arrow; (B) magnification of cell agglomeration in the crater-type structure.

of the beads showed that most of the cells were covered with a thin layer of alginate, which apparently did not inhibit the biotransformation of the substrate (Fig. 3A). At higher magnifications, the alginate gel shows quite large gaps in its microstructure, which may readily accommodate small substrates, oxygen and nutrients (not shown). Furthermore, several cell agglomerations are distributed over the surface of the bead, which cause crater-type structures in the gel (Fig. 3A), but these bacteria are still coated with a thin layer of alginate. It is interesting to note that some of the cells are in the process of cell division (Fig. 3B). This is in accordance with literature results, which describe the possibility of slow growth even under such restrictive conditions [30].

4. Conclusions

It was shown that a *B. megaterium* strain, isolated from topsoil, is capable of the chemoselective asymmetric hydroxylation of a large variety of hydrocarbons without further oxidation of the alcohol products. Furthermore, immobilization of the bacteria in alginate gel effectively increased the amount of products formed and prolonged the viability of the cells so that five catalytic cycles may be carried out. In addition, it was possible to upscale the free-cell batch fermentations to 2000 ml without significant differences to fermentations carried out on a smaller scale (75 ml). However, the improved productivity, as well as the easier handling, make immobilization the method of choice for this biotransformation. In general, the high enantioselectivity of this environmentally benign process encourages additional work to explore the scope of this biotransformation and further optimization for use at the preparative scale.

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