New Packing Materials for Bioreactors Based on Coated and Fiber-Reinforced Biocers

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Received March 2, 2004. Revised Manuscript Received May 12, 2004

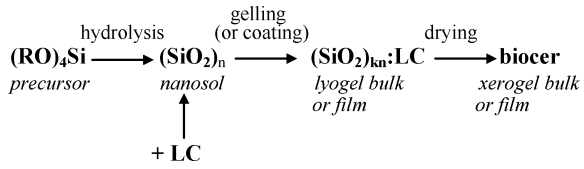
Stable shaped ceramic materials with embedded living cells that are applicable as biocatalytic packing material in bioreactors can be prepared by modification of the usual silica sol−gel process. The admixture of commercial alumina fibers (2−3 μm in diameter) shows an excellent reinforcing effect. By a simple casting process and drying in air, biocomposite materials with green-body character in any desirable shape are formed. The advantages of the biocers so prepared include high compactness, weak shrinkage during drying, and high mechanical stability. Due to the presence of residual water within the matrix, the incorporated cellular systems are living and show a high biocatalytic activity. Alternatively, biocatalytic packing materials can be fabricated by coating mixtures from aqueous silica nanosols with living cells on differently formed glass substrates. Coated and fiber-reinforced materials are successfully tested for the biodegradation of phenol with immobilized *Rhodococcus* cells and of glycol mixtures with *Aspergillus* spores in industrial wastewater.

1. Introduction

Silica and ceramic materials with embedded living cells (Biocers) are new composite materials that hold promise for applications in biocatalysis and bioremediation.[1−4] Biocers may be formed by using the recently developed sol−gel technology, which involves moderate temperatures and physiological conditions that enable denaturation and destruction of the sensitive living cells to be avoided, see Scheme 1. Some potential advantages of the inorganic ceramic materials as the host matrix for living systems are the excellent mechanical and thermal stability coupled with the possibility of controlling the porosity of the ceramic matrix over a wide range. Due to the matrix porosity, the embedded cells are easily accessible to external reagents and therefore biochemical reactions can proceed within the inorganic layer at a high rate. The ceramic-like matrix of biocers offers further advantages that ensure the survival of the embedded cells: the inorganic matrix is toxicologically and biologically inert, it is not a food source for microorganisms, and the preparation allows the additional incorporation of necessaries for life such as nutrients and humidity-stored compounds.

Scheme 1 illustrates a simple way in which the process of embedding the living cells (LC) in the sol−gel oxide matrix can be used to prepare bioactive layers and bulk products. During the gel formation the cells will be immobilized within the three-dimensional inorganic network. This is an essential step in the biocer formation. By hydrolysis and co-condensation with other metal alkoxide precursors (such as Al, Ti, and Zr compounds) biocers with various ceramic-like composition can be prepared.

Scheme 1. Biocer Preparation by the Sol−Gel Process Based on Silica (LC, Living Cells)



Recent alternative approaches to biocer preparation include freeze gelation[5] or special CVD processes with gaseous silans (*Biosil method*).[6−8]

Sol−gel immobilized microbial cells may become of great technical importance for pollutant biodegradation in water and soils. In recent investigations biotechnological destruction processes have been catalyzed with immobilized *Pseudomonas* strains for the biodegradation of the herbicide atrazine[9] and polychlorinated biphenyls[10,11] and with mixed bacteria and yeast strains for the continuous degradation of phenol in water.[10,12]

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10.1021/cm040156o CCC: $27.50
Published on Web 07/02/2004

To date, for the application of biocers in bioreactors the dried biocer bulks have been ground and classified to achieve an optimal grain size. Drying, grinding, and classification, however, are very expensive processes that unfavorably affect the embedded living cells, thus reducing the possibility of technical use.

Alternatively, coated biocers of mixtures from aqueous silica nanosols with living cells on different substrates can be prepared for use as packing materials in bioreactors. This cost-effective approach is only helpful if the biocatalytic activity is very high since the cell mass per reactor volume is much smaller compared with bulk products.

In an attempt to create a cheap biocer bulk material of a predetermined shape and moderate drying without subsequent grinding and classification, different solidification processes of the biocomponent-containing nanosol have been investigated. The successful preparation of form-stable fiber-reinforced biocers with embedded *Rhodococcus rhodochrous* (RC) cells and *Aspergillus versicolor* (AS) spores by means of alumina fibers, and their biocatalytic activity in bubble column bioreactors for the biodegradation of phenol and glycols in industrial wastewater compared with nonreinforced coated biocer material, are reported.

2. Experimental Section

2.1. Cultivation of the Biocomponents. The selection of the cell systems used was done following the requirements of industrial partners to develop new effective systems for the biodegradation of phenol and glycol mixtures in industrial wastewater. Microbiological studies revealed that *Rhodococcus rhodochrous* (RC) cells are very suitable for the biodegradation of phenol, whereas the halophilic fungus *Aspergillus versicolor* (AS) very efficiently biodegrades glycol mixtures in saline industrial wastewater.

Rhodococcus rhodochrous (German Collection of Microorganisms and Cell Cultures, DSMZ 6263) cells were grown in a 200-mL mineral medium (DSMZ media number 81) containing 500 mg/L phenol, in 500 mL flasks that were shaken at 30 °C. The cultivation was terminated at an optical density of 1.3. Cells were harvested by centrifugation at 3900 g for 5 min.

Aspergillus versicolor isolated from activated sludge of an industrial sewage plant (Leuna, Germany) was cultivated on nutrient agar (peptone15 g/L, yeast extract 3 g/L, NaCl 6 g/L, glucose 1 g/L, agar 12 g/L) supplemented with NaCl to a final concentration of 15%. For biocer coatings and bulk material AS spores were used. Spores were obtained by rinsing off with phosphate buffer, pH 7, from old cultures grown on nutrient agar.

2.2. Preparation of the Biocers. *(a) Biocer-Coated Glass Substrates*. Coated glass substrates were produced by using an AS or RC containing aqueous silica sol with a solid content of 4.2 wt % and a mean particle size of 6 nm (by Zetasizer 1000 HS/Malvern). The sol was prepared using acid catalyzed hydrolysis of tetraethyl orthosilicate (TEOS).[13] To obtain an aqueous silica sol, ethanol was evaporated by leading air through the solution simultaneously substituted by water. Before adding the biocomponent to the silica sol the pH was increased up to pH 7 by adding 1-M NaOH. Pore forming monosaccharides,[14,15] such as Sorbitol (up to 50 wt %/wt SiO_2) were used in RC-containing sols as an additive to achieve higher porosity. The composite sol with AS spores or RC cells was dip-coated on either glass debris with a middle particle size of 10 × 10 mm (AS−C) or small glass cylinders of a length and diameter of 15 mm each (RC−C). The amount of biocomponents was adjusted to get a cell density of approximately 10^6 RC cells/cm^2 or 2×10^6 AS spores/cm^2.

(b) Fiber-Reinforced Biocer. For the fiber-reinforced biocer material alumina fibers (diameter 2−3 μm, VWR) and silica sol (Nyacol 1440, Akzo Nobel) were mixed up to a viscous consistency. After adding the biocomponent and casting, the biocer was dried for 24 h. 1 g of the AS biocer (AS−R) contained 4.5×10^7 spores. In the case of RC−R biocers the amount of cells was 7.6% (mass wet RC cells/wt biocer).

2.3. Characterization of Biocers. *(a) Porosity*. To determine the pore distribution of the fiber-reinforced biocer material, gas adsorption and mercury intrusion were used. The specific surface area and related pore volume were measured by gas adsorption (ASAP 2010, Micromeritics) using nitrogen. Mercury intrusion experiments (Pascal 140 and 440, Porotec) were carried out to evaluate the pore diameters in the range of 4 nm to 100 μm.

(b) Microscopy. Fluorescence microscopy was used to detect viable cells embedded in silica coatings after staining with the LIVE/DEAD *Bac*Light kit (Molecular Probes, Leiden, Netherlands).

SEM micrographs were taken using a Gemini 982 scanning electron microscope (LEO, Oberkochen) with an energy-dispersive X-ray analyzer (NORAN X-ray detector) at 1−5 kV. Biocer samples were prepared by embedding in liquid colloidal silver on conductive carbon sheets and shadow casting was made with carbon (Baltec MED 010, BAL-TEC, Liechtenstein).

(c) Mechanical Examination. Measurements of the tensile strength of the shaped fiber-reinforced biocers were determined by diametral compression using the tablet tester PTB-311 (Pharmatest) applying the equation: $T = 2F/\pi dt$. T is the tensile strength, F is the force (N) needed to cause fracture, d is the diameter, and t is the thickness of the biocer body.

2.4. Biodegradation Experiments. Phenol degradation was examined under fed-batch conditions in an aerated bubble column bioreactor filled with 450 g of coated glass cylinders resulting in a liquid volume of about 250 mL. A starting concentration of 500 mg/L phenol was used, and this was increased by 500 mg at each cycle to a final concentration of 3000 mg/L. The phenol concentration was measured colorimetrically at 500 nm after dye formation with 4-aminoantipyrene and potasiumhexacyanoferrat of 1 mL samples.[16]

Biodegradation experiments with immobilized AS spores were carried out using a synthetic saline wastewater (DSMZ mineral medium 81 supplemented with 0.1% yeast extract, 0.5% glucose, 0.1% casein, x% xenobiotic stock solution, and NaCl to a final concentration of 15%). Different xenobiotic concentrations, x%, were tested therein for concentrations up to 40% of the stock solution (8 g/L methanol, 4 g/L ethylene glycol (EG), 1 g/L diethylene glycol (DEG), 8 g/L ethylene glycol monomethyl ether (EGMME), 1 g/L ethylene glycol dimethyl ether (EGDME), 2 g/L diethylene glycol monomethyl ether (DEGMME), 0.5 g/L diethylene glycol dimethyl ether (DEGDME), and 0.5 g/L triethylene glycol monomethyl ether (TEGMME)). No inhibitory effect was observed. Gas chromatography (Hewlett-Packard 5890 Series II, 30 m × 0.32 mm column packed with ZB−WAX, N_2 as the mobile phase) with a FID detector was undertaken to determine the concentration of the different glycols and glycol ethers. Investigations were carried out in 2-L bubble column bioreactors with a packed bed volume of approximately 1.3 L under batch conditions at room temperature. The aeration was accomplished with sterile air.

3. Results and Discussion

3.1. Coated Biocer Material. Until now, the creation of formstable packing biocer materials for applica-

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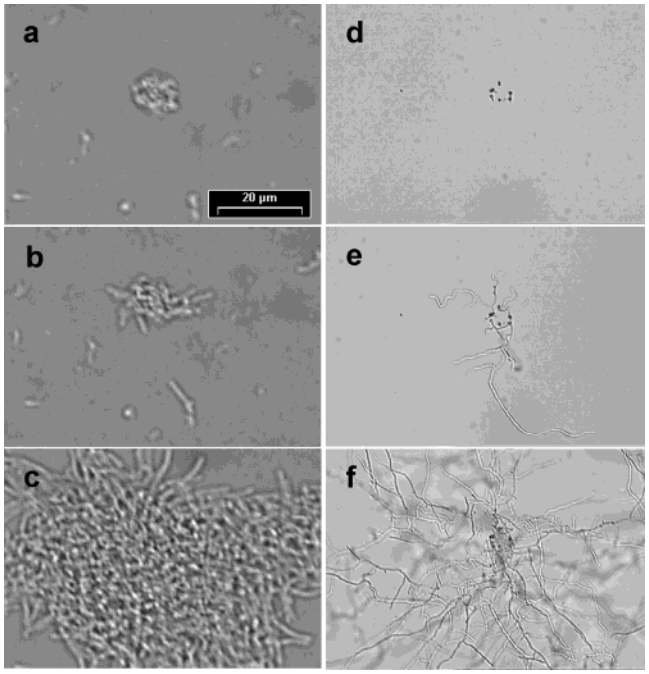

Figure 1. Evolution of embedded *Rhodococcus rhodochrous* cells and *Aspergillus versicolor* spores after incubation in nutrient broth (a−c), growing RC cells after 4,10, and 22 h feed contact; (d−f), evolution of AS-spores after 0, 10, and 20 h.

tion in bioreactors has been accomplished by embedding of the living cells via the standard sol−gel process according to Scheme 1. For the preparation of coated glass or other substrates it is necessary to use aqueous nanosols (as described by Soltmann et al.[13]) with small, mostly nonaggregated particles (mean size < 10 nm) that show excellent adhesion and film formation properties on different substrates. Using such silica nanosols, the coated samples RC−C (with *Rhodococcus spec.* cells) and AS−C (with *Aspergillus versicolor* spores) on industrial glass debris were prepared, see Figure 1. The thickness of the silica layer was about 240 nm by optical measurements.

The viability of the embedded RC cells was proved by the phenol degradation rate and, in addition, by staining with SYTO 9 and propidium iodide to detect the membrane integrity (Figure 2). The "Live/Dead" staining method results in approximately 70% of the embedded cells remaining alive after a storage time of 3 days at 4 °C. After a period of 5 days only 10% of the cells are living, decreasing to 5% after 9 days.

For immobilization of AS within thin silica layers the voluminous mycel of the fungus is unsuitable. Therefore, spores of AS were used. The viability of the AS spores can be seen by budding after feed contact (see Figure 1 d−f). To rule out the possibility that the spores on the layer surface are responsible for the budding effect and to ensure a good immobilization of the large spores, AS−C samples were additionally coated up to 3 times with an inert silica layer, thereby allowing us to also prove the occurrence of budding within the layer. It should be mentioned that of all cell systems spores are most suitable for biocer preparation. Biocers containing spores are long-term stable (for example more than 30 months in the case of AS spores) and less sensitive to preparation, drying conditions, and water content.

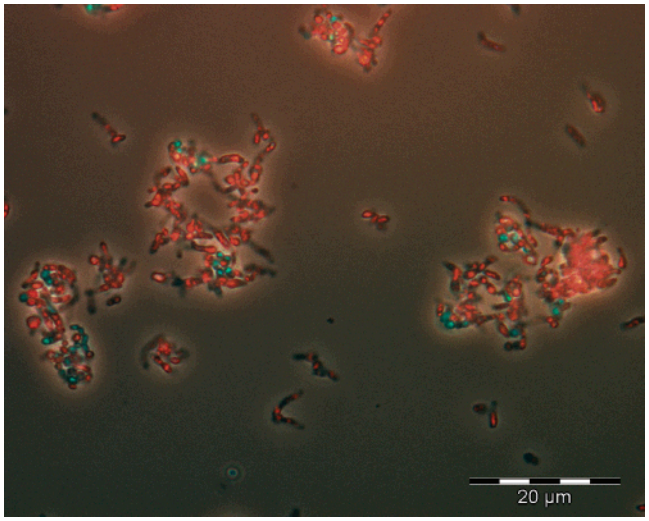

Figure 2. Fluorescence micrograph of RC cells embedded in a silica layer after a storage time of 9 days. 'Live/Dead' staining with SYTO 9 and propidium iodide (green = living cells, red = dead cells).

Embedded spores also retain their ability to germinate in dry biocers, and their reactivity is often comparable to that of the nonspored cell system.

3.2. Fiber-Reinforced Biocer Material. As an alternative to the coated samples, RC and AS were embedded in ceramic bulk materials by modifying the usual sol−gel process. For this purpose, the following modifications were introduced:

(i) Using high-concentrated aqueous silica nanosols. Such sols with mean particles > 10 nm are commercially available, for example, Ludox/Grace, Nyacol/Akzo Nobel, Levasil/Bayer (it should be noted that they are not quite suitable for coating processes because adhesion and film formation properties diminish with increasing particle size).

(ii) The addition of commercial alumina fibers (diameter 2−3 μm) to ensure form stability after gelation (reinforced effect).

After the mixing of nanosol, alumina fibers, and the biocomponent, various forms with green-body character can be produced by a simple casting process, gelling, and drying in air. Surprisingly, the addition of alumina fibers causes strong strengthening of the gel matrix although the fibers are randomly distributed within the composite matrix, see Figure 3. In this way, the shaped biocers RC−R and AS−R could be prepared and used further as packing material in bioreactors, see Figure 4c,d.

The optimum weight ratio of silica to alumina fibers is 1:0.5−1. To increase the solid content, alumina powder (e.g., with a mean particle size of 700 nm) up to 30 wt % can be added. Normally, the content of the cell mass of the biocer is below 5 wt % since higher concentrations reduce the stability in contact with water. In the case of higher cell mass contents the stability of the shaped packing material can be improved by post-treatment and infiltration with pure silica nanosols. In this way biocers with cell mass contents up to 20 wt % were realized. In the case of vegetative cells such as RC the presence of residual water within the biocers is required for long-time bioactivity. To that end, the addition up to 16 wt % glycerol is advantageous,

Figure 3. SEM micrograph of an AS−R biocer body.

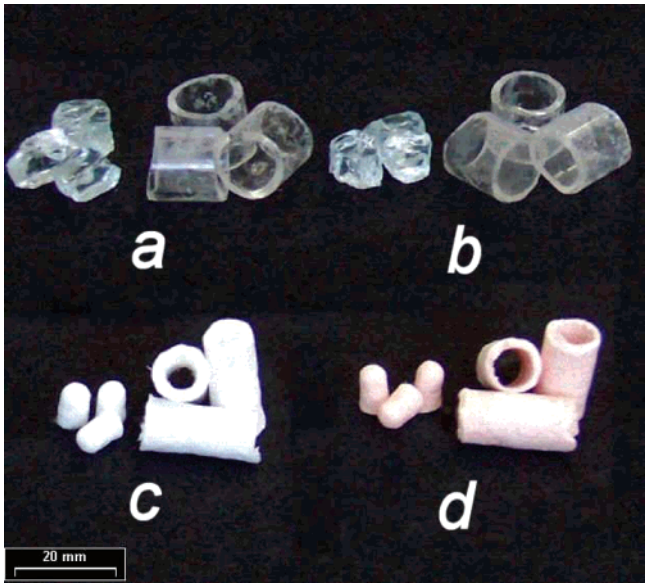

Figure 4. Pictures of the prepared packing materials based on coated and fiber-reinforced biocers: (a) AS−C, (b) RC−C, (c) AS−R, and (d) RC−R.

because polyols such as glycerol (or PEO, PVA) act as a matrix softener and humidity retainer, which suppress the cell lysis and extends considerably the viability of the embedded cells.[17] Co-immobilized sorbitol is also known to enhances the bioactivity due to its pore-forming properties after leaching before or during its use as a biocatalyst.

The new fiber-reinforced biocers such as RC−R and AS−R show a high mechanical stability and compactness, weak shrinkage during drying, and high long-term stability during the application in biorectors, when the share of the biocomponent is not too high (less than 5 wt %). Compact biocer bodies (Figure 4) possess a tensile strength of 2.34 MPa in the case of AS−R and 1.89 MPa for RC−R biocers. As expected, biocers shaped as hollow cylinders show a diminished tensile strength of 0.12 MPa (AS−R) and 0.03 MPa (RC−R). Furthermore, the biocers exhibit a bimodal distribution of macroporous and mesoporous structures, as shown in Table 1 and

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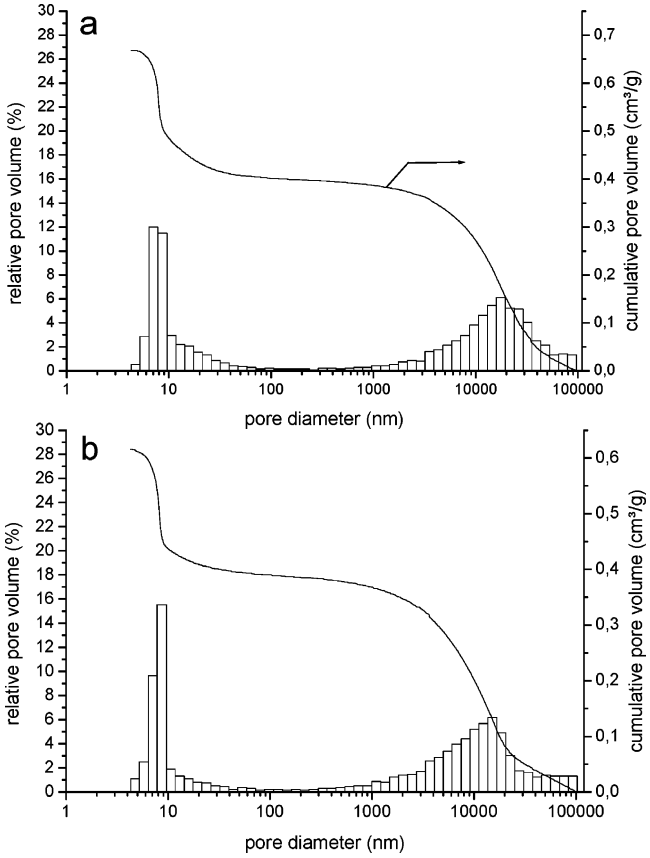

Figure 5. Pore distribution of fiber-reinforced biocers with (a) AS spores or (b) RC cells as biocomponents.

Table 1. Pore Structure of Fiber-Reinforced Biocers with *Aspergillus versicolor* Spores (AS−R) or *Rhodococcus rhodochrous* Cells (RC−R)

		AS−R	RC−R
gas adsorption	BET surface area (m^2/g)	131.5	113.1
	BET average pore diameter (nm)	9.31	9.26
	single point total pore volume (cm^3/g)	0.3058	0.2619
	BJH desorption cumulative pore volume (cm^3/g)	0.2961	0.2522
mercury intrusion	total cumulative volume (cm^3/g)	0.6683	0.6163
	total porosity (%)	60.2	54.6

Figure 5, allowing good evaluation of embedded biocomponents to be done. Due to the presence of residual water within the matrix, the incorporated cellular systems are living and show a high biocatalytic activity.

3.3. Biocatalytic Properties. The biocatalytic activity of the biocer materials was tested with regard to the degradation of phenol and glycol ethers to different lower molecular aliphatic compounds and carbon dioxide. The tests were carried out over a period of 3 months, wherein no decrease in the activity of the used biocer material was observed.

The phenol degradation was achieved by biocers containing living *Rhodococcus spec.* cells in a bubble column reactor. Non immobilized RC cells can use phenol up to a concentration in excess of 2.5 g/L as a single carbon and energy source under batch conditions. After immobilization, phenol concentrations up to 3 g/L were tolerated and degraded.

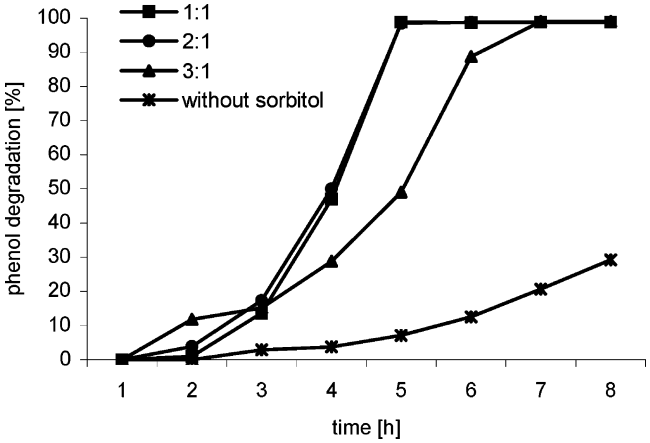

Figure 6. Activity of immobilized RC cells coated on glass (RC−C) in dependence on different amounts of sorbitol used for the preparation of the composite sol (weight SiO_2: sorbitol).

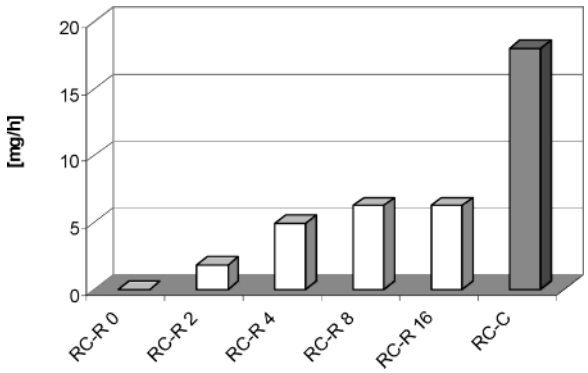

Figure 7. Biodegradation of phenol in a bubble column reactor packed with RC−C or RC−R (0, 2, 4, 8, 16% glycerol).

The phenol utilization was studied as a function of residual water content of the silica matrix and pore forming additives, and also as depending on the way of preparation. For coated biocer materials sorbitol was used to enhance the activity of the embedded RC cells (see Figure 6).

Best results for fiber-reinforced biocers were achieved by adding 16% glycerol to the slurry. Figure 7 shows the influence of the glycerol content on bioactivity. Comparing bubble column reactors filled with RC−C or RC−R biocer material, a 3-times higher phenol degradation rate was observed using coated glass cylinders. The lower activity of the RC−R biocers is caused by a longer and more unfavorable experimental procedure. In agreement with this, by using the considerably more robust spores as a biocomponent for RC−R biocers, a clearly smaller impairment by the production process is shown, see Figure 8.

Aspergillus spores embedded within the silica very efficiently degrade different glycols in salt-containing industrial wastewater. To ensure high degradation rates, additional nutrients such as glucose or casein and yeast extract are required. Comparison between the use of biocer-coated glass bodies and fiber-reinforced packing material, as shown in Figure 8, reveals the high bioactivity of the reinforced biocer in a column bioreactor.

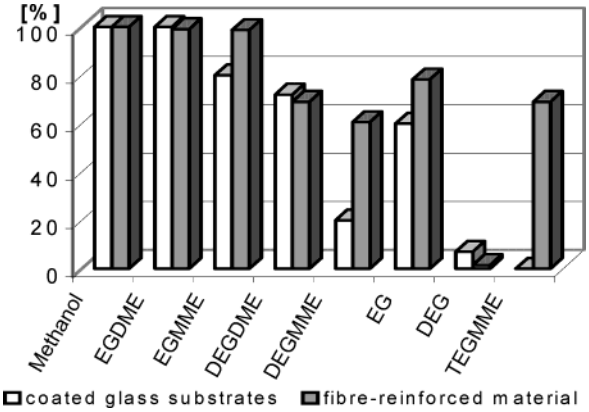

Figure 8. Biodegradation of different glycols after 21 days in a bubble column reactor packed with (a) AS−C and (b) AS−R.

4. Conclusions

By combining silica sols with alumina fibers, new forms of biocers can be obtained, thus allowing the preparation of stable shaped bulk products. The advantages of the new fiber-reinforced biocers are their high compactness, weak shrinkage during drying, and high stability when the fraction of the biocomponent is not too high. Due to the presence of residual water within the matrix, the incorporated cellular systems are living and show a high biocatalytic activity. Since the amount of biocomponent for biocatalytic purposes can be very small, one can produce very cheap compositions by additional admixture of alumina powders. Both coated and fiber-reinforced biocers are active biocatalysts and are well suited for biodegradation of phenol with immobilized *Rhodococcus* cells and of glycols with *Aspergillus* spores in industrial wastewater. The positive results obtained indicate that biocers have great potential for widespread industrial utilization in the field of industrial wastewater recycling.

Acknowledgment. Financial support of the German Ministry for Education and Research (Project 02WA0134) and the German Research Community (Project Bo 1070/4-2) is highly acknowledged. We would like to thank the Department of Ceramic Materials and Components, University of Bremen, for help in performing the surface area and pore distribution measurements and Dr. Schmidt-Brücken (Institute of Material Science, Technical University of Dresden) for the SEM micrographs.

CM040156O