CHEMISTRY OF MATERIALS

VOLUME 16, NUMBER 26 DECEMBER 28, 2004

Communications

Calcium Dipicolinate Induced Germination of *Bacillus* Spores Embedded in Thin Silica Layers: Novel Perspectives for the Usage of Biocers

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Received August 24, 2004
Revised Manuscript Received October 26, 2004

The unique advantages of biological ceramics (biocers) as a fascinating combination of a robust and stable silicate matrix with several kinds of sensitive biologics at physiological conditions became an increasingly interesting subject for the development of application-directed or tailored materials in sensor technology, medicine, biotechnology, and environmental technology.[1−5] Meanwhile, numerous publications described several promising ways for the immobilization of whole cells or cell-derived subunits while retaining their viability or functionality up to several weeks.[6−9] Thereby, the content on cytotoxic alcohol in the reaction mixture as well as the drying regime influence dramatically the membrane integrity of the embedded cells.[10] To overcome these problems different strategies to preserve the cell integrity are available: keeping the alcohol content as low as possible by using aqueous precursors,[11] addition of preserving agents such as glycerol or poly(vinyl alcohol),[12−14] keeping the gel in a wet atmosphere,[15] or minimization of undesirable mechanical stress impact caused by shrinkage effects upon drying by using modified "low-shrinkage" precursors.[12]

However, the insufficient long-time viability of the embedded cells remains a critical point in the fabrication of biologically modified sol−gel ceramics and thus potentially limits the applicability for biotechnological requirements.

To control and preserve the bioactivity of bacterial cells in silica gels therefore still represents a real challenge. In this work the fitness of immobilized

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10.1021/cm040191v CCC: $27.50
Published on Web 11/23/2004

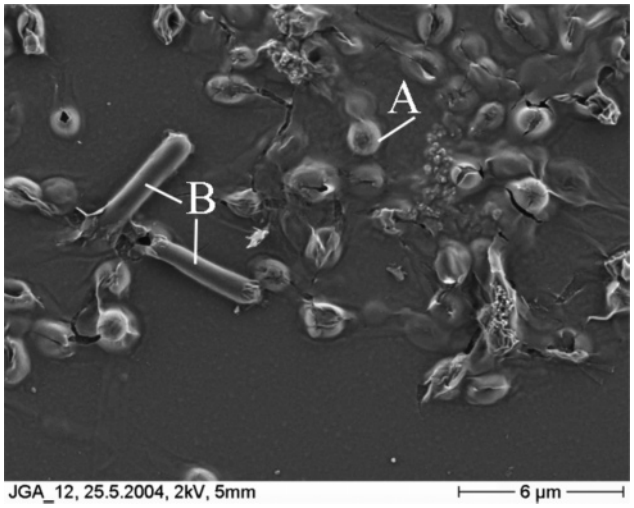

Figure 1. Scanning electron microscopic image of sol−gel immobilized *B. sphaericus* JG-A12 spores activated with 60 mM Ca^{2+}−DPA and fed with NB for 4 h: (A) spore before germination, and (B) outgrown vegetative cells.

Table 1. Viability of Sol−Gel Immobilized Vegetative Cells of *B. sphaericus* JG-A12

storage time	storage conditions	living cells
fresh prepared	wet, room temp.	100%
72 h	dry, 4 °C	18%
	wet, 4 °C	32%
	wet, room temp.	17%

vegetative *Bacillus* cells and spores in thin sol−gel layers was compared. As a result of these investigations, a novel approach to affect the germination of immobilized spores will be described.

The Gram-positive soil bacterium *Bacillus sphaericus* JG-A12, an isolate from a uranium mining waste pile near the town of Johanngeorgenstadt in Saxony, which is able to bind large amounts of heavy metals and radionuclides, was chosen as the biocomponent.[16] These favorable characteristics make it a good candidate for bioremediation studies. Silica sol−gel filter materials with embedded *B. sphaericus* JG-A12 cells have already shown a remarkable potential for removal of uranium and copper from uranium mining wastewaters.[17,18]

Thin films of silica gels containing vegetative cells of *B. sphaericus* JG-A12 were prepared by dip-coating glass slides at a constant pulling rate of 300 mm/min in an aqueous silica nanosol produced by acid hydrolysis of tetraethyl orthosilicate (TEOS).[18] The glass surface was prehydrophilized by incubation in deionized water for 48 h at 70 °C. Immediately after film deposition the glass slides with vegetative cells were transferred in a wet atmosphere and stored at 4 °C or at room temperature. The intactness of the cell membranes was controlled by Live/Dead staining.[19] As seen in Table 1, the number of viable cells decreased dramatically in less than 3 days independent of the storage conditions. Air drying of the cell-containing gel layers for 30 min provoked membrane damage of the complete bacterial population.

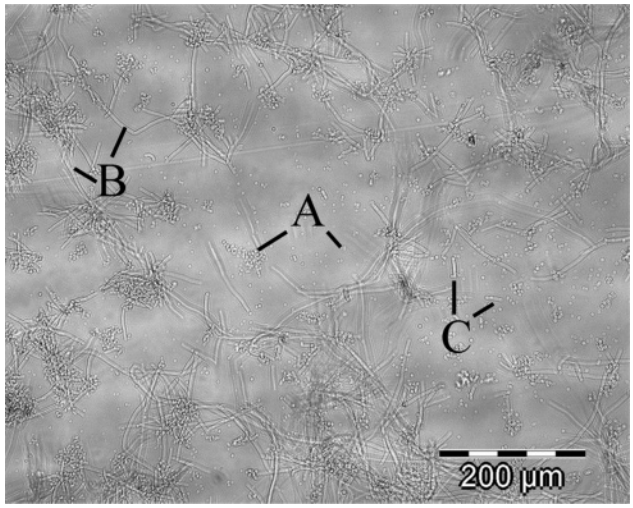

Figure 2. Light microscopic image taken 15 h after induction of the spore germination in a 2.5-year-old silica layer: (A) spores, (B) enlarged vegetative cells of early germinated spores (3−4 h after the induction), and (C) outgrowing vegetative cells from late-germinating spores.

Advantageously, the strain JG-A12 possesses the capability to sporulate very easy and thus offers a simple way to generate biocers suitable for long-time storage without consideration of any specific nutrition conditions. Due to the fact that dormant spores contain practically no water it should be possible to store the biocomposites under dry conditions for a long period of time. To prove the germination capability of the embedded spores two kinds of thin films were prepared: pure aqueous silica gels and gels supplemented with 20% sorbitol to improve their porosity. The gel-covered glass slides were held in dry or wet atmosphere at 4 °C and at room temperature. At different intervals the slides were assembled in a flow-through chamber and fed with nutrient broth (NB)[20] at a constant flow rate of 0.4 mL/min. Surprisingly, the germination rate after 24 h of feeding remained below 10% even for fresh-prepared layers although more than 90% of the spore suspension was able to grow out on agar plates. It was demonstrated in the literature that in the case of the nutrient-dependent germination, the secretion of calcium and dipicolinic acid (Ca^{2+}−DPA) from the activated spores plays a key role.[21] Ca^{2+}−DPA can induce spore germination also in an alternative, nutrient-independent way.[22−25] To check the effect of Ca^{2+}−DPA on sol−gel immobilized *B. sphaericus* spores, sorbitol containing slides were flooded with 60 mM Ca^{2+}−DPA[26] for 1 h and subsequently fed with NB for 2 days. The first outgrowing cells could be observed already after 3.5 h

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(19) The slides were incubated for 5 min with 5 μM SYTO 9 and 55 μM propidium iodide in deionized water.
(20) The NB medium was prepared with 3 g/L of meat extract and 5 g/L of peptone, and supplemented with 10 mg/L of manganese sulfate.
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(26) The chamber was filled with 120 mM $CaCl_2$ solution and subsequently diluted with 120 mM DPA solution at a constant flow rate for preventing any precipitation of the chelate. Both chemicals were dissolved in 10 mM Tris buffer and adjusted to pH 8.

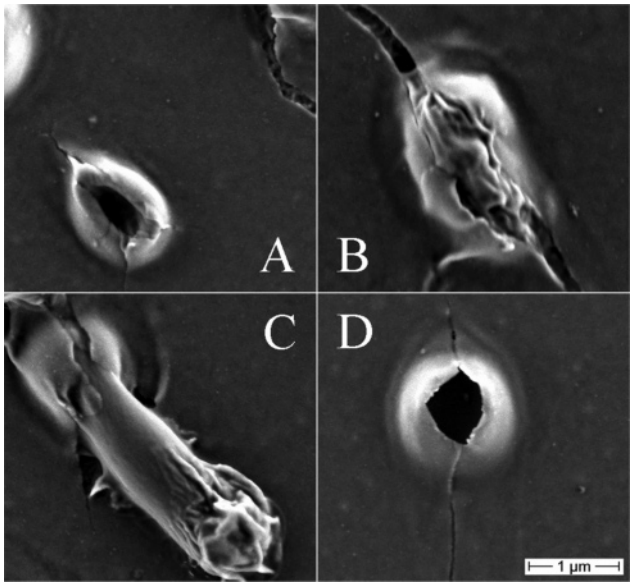

Figure 3. Germination phases of immobilized *B. sphaericus* JG-A12 spores: (A) inversion in the early phase, (B) germinating spore, partial breaking of the silica layer, (C) outgrowing vegetative cell, and (D) silica layer after cell release.

(Figure 1). The overall effect of the Ca^{2+}−DPA treatment was a 5−8-fold enhanced germination rate independently of the pore-forming agent sorbitol. The same behavior was observed with 2.5-year old silica layers stored at room temperature under dry conditions (Figure 2). Neither $CaCl_2$ nor DPA alone could induce this accelerated and enhanced outgrowth of spores.

The stimulating effect of external Ca^{2+}−DPA in conjunction with the poorly developed germination behavior of the spores in the presence of nutrients indicates that only the nutrient-specific mechanism of the germination induction is inhibited by the immobilization process.

Noticeably, the silica layers maintain a respectable mechanical stability during the germination process. Figure 3 illustrates the resilience of the layer during the single phases of germination.

This mechanical robustness combined with the possibility to induce a sufficient and tunable outgrowth by the chemical trigger Ca^{2+}−DPA opens new perspectives for bacteria-based biotechnological applications in view of bioreactor design and process controlling.

In this way, the stepwise modulation of the active biomass in dependence on the specific requirements in different fermentation processes becomes a conceivable vision.

Furthermore, the inhibition of the nutrient-inducible system by immobilization of spores in silica matrixes prevents any undesired outgrowth. Considering safety aspects during handling of microorganisms, and in particular, if they are genetically modified, the opportunity to control and regulate the germination process by a chemical inducer could be an interesting ancillary tool with practical relevance.

Acknowledgment. This work was supported by grants DFG PO 392/15-2, SE 671/7-2, and BO 1070/4-2 from Deutsche Forschungsgemeinschaft, Bonn, Germany

CM040191V