

Microcultivation of anaerobic bacteria single cells entrapped in alginate microbeads

Rosa Aragão Börner · Maria Teresa Alvarez Aliaga · Bo Mattiasson

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Abstract Alginate microbeads, produced by emulsion/internal gelation, were studied for the entrapment and microcultivation of microbial cells with biotechnological potential. An anaerobic consortium which was selected for its capacity to degrade complex carbohydrates, and a pure culture of cellulose degrading bacteria were used for entrapment studies. Optimization of conditions for the formation of spherical alginate microbeads in sizes between 20 and 80 μm were examined. The best conditions were achieved by combining rapeseed methyl ester as oil phase and stirring at 100 rpm using a rotation impeller. Calcium alginate microbeads produced under these conditions were shown to present morphological stability, with

large pores in the internal matrix that favours microcolony development. Finally, single cells were observed inside the beads after the entrapment procedure and microcolony formation was confirmed after cultivation in cellobiose.

Keywords Alginate · Anaerobic bacteria · Single cell · Microcultivation

Introduction

Immobilization of microbial cells by entrapment has been widely used in industrial and scientific applications for many years. Various materials have been used for the entrapment of microorganisms including alginate, agarose, collagen and chitosan. In addition to its biocompatibility, the use of alginate offers the advantage of being an extremely mild process in which a solution of sodium alginate together with the cells of interest comes into contact with calcium ions which cause the negatively charged alginate to gel, forming a polymer capsule surrounding the cell. The use of such materials for the entrapment of microbial single cells for further microcultivation has however not been fully exploited.

Currently, different methods are used to entrap cells in alginate. The most traditional procedure is by extrusion of the sodium alginate solution in CaCl_2 solution. However, microparticles of less than 500 μm

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R. A. Börner · M. T. A. Aliaga · B. Mattiasson (✉)
Department of Biotechnology, Center for Chemistry
and Chemical Engineering, Lund University, PO Box 124,
SE-22100 Lund, Sweden
e-mail: Bo.Mattiasson@biotek.lu.se

R. A. Börner
e-mail: Rosa.Aragao@biotek.lu.se

M. T. A. Aliaga
e-mail: materesa_alvarez@yahoo.com

M. T. A. Aliaga
Instituto de Investigaciones Farmaco Bioquímicas,
Facultad de Ciencias Farmaceuticas y Bioquímicas,
Universidad Mayor de San Andrés, La Paz, Bolivia

are difficult to obtain with this method. They tend to be teardrop-shaped and there is also a significant degree of clumping of the microspheres. On the other hand, in the emulsion/gelation process, insoluble calcium citrate micro-crystals serve as an internal calcium source. The mixture is emulsified in a hydrophobic medium and, due to a pH reduction; calcium is released, forming Calcium alginate microbeads. Two main advantages are obtained with this technique: (1) formation of relatively homogenous beads (Quong et al. 1998) and (2) a wide range of sizes of microspheres can be obtained by means of controlling the conditions of the water-in-oil emulsion (Poncelet 2001).

The emulsion/gelation method has been used to entrap various biological compounds such as proteins (Reis et al. 2007), DNA (Quong et al. 1998), bacteria (Rodrigues et al. 2006) and yeast (Serp et al. 2000). It has also been used to increase cell density of wastewater activated sludge for in situ bioaugmentation of contaminated aquifers (Moslemy et al. 2004). To our knowledge, this technique has not been applied to the entrapment of single cells of strict anaerobic pure culture or consortia.

The emulsion-gelation technique with alginate as matrix gel provides several advantages for entrapment/cultivation of anaerobic consortia for single cells. It is simple and easily performed inside an anaerobic chamber and it is biologically safe. These conditions make the technique especially suitable for anaerobic microorganisms that are more sensitive to slight changes in the environment for their growth.

For a more efficient application of alginate microbeads produced by emulsion/gelation, morphological characteristics such as size and shape are important. As the volume of a microbead becomes smaller, the probability of obtaining one cell per bead becomes greater (Weaver et al. 1988). For this reason our aim was to obtain microbeads between 20 and 80 μm in diameter. Mean diameter and shape of the beads can be controlled by changing the emulsification conditions (Reis et al. 2006) such as type of oil and mechanical shear. The present study was carried out in order to optimize the conditions during emulsion for the microentrapment of single cells from an anaerobic consortium by an internal-gelation procedure that allows further microcultivation of the cells.

Materials and methods

Materials

Sodium alginate from brown algae, medium viscosity (2 % w/v $\geq 2,000$ cps at 25 °C) was used as gel matrix and was purchased from Sigma. Rapeseed methyl ester 99 % (RME) was kindly provided by Perstorp Oxo AB (Stenungsund, Sweden), rapeseed oil (RPS) (Zeta) was obtained from local supermarket. Calcium citrate was obtained from Aldrich and Tween 85 and glacial acetic acid were also purchased from Sigma.

Microorganisms and culture conditions

Anaerobic consortia and *Clostridium sulfatireducens* CCUG 50825 were used for the encapsulation procedure. Consortium A10 sample was collected from the shores of the lake, Laguna Challviri (67°38'W, 22°, 32' S), and consortium L1 was collected from the river Lakajahuira (67°4'W, 19°11'S). Both locations are situated in the Bolivian Andean region. *C. sulfatirreducens* CCUG 50825 was previously isolated from consortium A10 (Alvarez et al. data not published).

The consortia and *C. sulfatirreducens* were cultivated in modified Pfennig medium (Pfennig et al. 1981) with no addition of volatile fatty acids and replacing sodium dithionite by cysteine-HCl as reducing agent. Different material of cellulose/hemicellulose origin were tested as carbon source for selection of consortia and 3 g cellobiose l^{-1} was used for cultivation of cells entrapped in calcium alginate microbeads. The culture broth was placed into anaerobic serum vials and flushed with O_2 -free N_2 according to the serum bottle modification of the Hungate technique for cultivating obligate anaerobes.

Anaerobic consortia selection

The consortia were tested for their capabilities to degrade cellulose/hemicellulose material, by their growth on different carbon sources. Modified Pfennig medium was used as described above and glucose, starch, maltose, lactose, xylan, cellobiose, carboxymethyl cellulose (CMC), at 1 g l^{-1} , and 1.5 g l^{-1} (2 filter paper strips) were used as carbon source. Cultures of sample consortia A10 and L1 were incubated at 37 °C and 30 °C, respectively. Turbidity

was observed in the vials after 15 and 30 days of cultivation.

Entrapment of anaerobic cells in alginate by emulsion/gelation technique

For microcultivation in an alginate bead, it is necessary to limit entrapment to one cell per bead in most cases. For a higher probability of initial entrapment of one single cell, a low volume for each alginate bead must be considered. Estimating 50 μm as an average size for each alginate bead, the initial cell concentration, 2.04×10^6 cells/ml, was based on Poisson distribution as suggested by Weaver et al. (1988). Cells of *C. sulfatirreducens* and of the consortium selected from the screening process were grown in modified Pfenning medium and serially diluted to reach the desired initial cell concentration.

Alginate microbeads were prepared by emulsification/gelation (Poncelet et al. 1992). Preparations of oil and solutions, as well as the entire entrapment procedure, were carried out under oxygen-free nitrogen conditions inside an anaerobic chamber (Plas-Labs, USA).

A sterile 2 % (w/v) sodium alginate solution was prepared by adding sodium alginate particles, which were sterilised for 1 h under UV in a sterile cabin, into autoclaved de-ionized water to avoid degradation of the alginate by the autoclave high temperature (Chen et al. 2005). The solution was stirred overnight in a closed bottle. Sterility was confirmed by no growth observed on agar plates, when solution was inoculated as a test.

The sodium alginate solution was mixed with ultrafine calcium citrate (500 mM Ca^{2+} , sonicated for 2 h) slurry at 13,500 rpm with a homogenizer (Ika-Werke Yellow line DI 25 basic, Germany) for 2 min. The cell suspension was added (1:1) and mixed for 2 min with the homogenizer. The alginate-cell gel was poured into a beaker containing the hydrophobic phase and mixed with a mechanical head stirrer with a two-blade 25 mm impeller for 15 min as emulsification time in a ratio of 1:20. Still stirring, more hydrophobic phase containing 80 μM acetic acid was added to the water/oil emulsion for 2 min. By doing this, the pH of the system was reduced to approx. 5. Oil dispersed microspheres were recovered with a solution of 50 mM CaCl_2 by shaking with the emulsion system for 1 h at room temperature at 130 rpm.

Mixture of oil and beads/ CaCl_2 was serially centrifuged at $2,000 \times g$ for 5 min to remove oil phase as much as possible. A final concentration of 0.1 % (v/v) Tween 85 was used to wash the beads once and microbeads were successively washed with a mixture of Tris-HCl buffer 50 mM pH 7.5 and CaCl_2 until no more oil was detected under optical microscope observation. Finally, microspheres were kept in Tris/HCl buffer 50 mM pH 7.5 at 4 °C for further studies.

Optimization of alginate bead shape and size

The optimal conditions for single cell entrapment in calcium alginate by emulsion/gelation technique based on microsphere shape and size were determined using factorial design experiments. Three independent variables of the process for the bead formation were tested: (1) hydrophobic phase: rapeseed oil (RPS) and rapeseed methyl ester (RME), (2) presence of baffles or not in the beaker where the reaction occurs and (3) rotation speed when mixing: 100 or 1,000 rpm. The combination of each of these factors was performed and samples were stored at 8 °C for measurement and characterization.

The shape and diameter of the microspheres were determined by optical microscopy equipped with a digital camera AxioCam MR3 and an AxioVision Rel. 4.7 image analyser (Carl Zeiss, Germany). Calibrated tools for determination of shape and size from the image analyser software were used.

To evaluate the results from the experiments, thirty calcium alginate beads were considered for each batch test of the combination of the three variables, which were done in triplicate. Only the alginate beads that showed a real bead shape (spherical) were considered to be measured for size determinations. Factorial ANOVA was used to analyse the importance of the three variables in regards to size and shape of microspheres. The analysis was done using the software STATISTICA v9.12 and graphics were plotted using MATLAB vR2009b.

Alginate microbead morphology characterization

Calcium alginate microbeads were investigated by scanning electron microscopy (SEM) to identify morphological characteristics of importance for further microcultivation application. Microbeads were treated for 1 h in a 1 % (w/v) OsO_4 in distilled water,

and then rinsed twice with distilled water. Thereafter, they were dehydrated gradually in serial ethanol solutions of 50 % (10 min), 70 % (twice), 96 % (twice) and finally absolute ethanol. The dehydrated microspheres were then CO₂ critical point dried in a CPD 030 (BalTec, Liechtenstein). The dried microspheres were mounted on stubs and coated with gold/palladium with a SC7640 sputter coater (Quorum Technologies, Newhave, East Sussex, UK). Scanning electron microscopy was carried out with a JSM-5600LV microscope (Jeol Ltd., Tokyo, Japan) with acceleration voltages between 6 and 10 kV.

Entrapment of single cells and microcultivation of entrapped cells

Calcium alginate beads were observed using bright field and phase contrast microscopy just after the entrapment preparation and after cultivation in modified Pfenning medium with 3 g l⁻¹ cellobiose as carbon source.

Results and discussion

Anaerobic consortia selection

The microorganisms of both consortia degraded almost all carbon sources tested, the main difference between them being the consumption of filter paper. Observation of the turbidity of the broth and the appearance of the substrate revealed that the two consortia can use both simple and complex carbon sources: glucose, lactose, maltose, starch, xylan and cellobiose. The vial test with medium containing CMC did not show dense turbidity even after 30 days of cultivation. However, the main difference was the consumption of filter

paper, where degradation of the substrate was observed for consortium L1, but not for consortium A10.

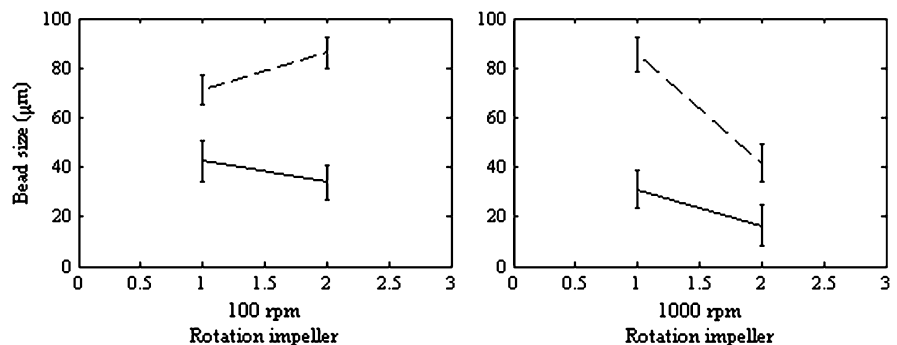
The ability of consortium L1 to degrade filter paper is of particular biotechnological interest. The finding shows that L1 contains microorganisms able to degrade polymeric substrates such as cellulose and hemicellulose, two of the most abundant renewable carbon sources on earth. The degradation of these materials is thus of high interest for biotechnological transformations (Viamajala et al. 2010). By its ability to metabolize not only simple but also complex carbon sources (filter paper), L1 consortium was selected for entrapment tests.

Optimization of alginate beads for size and shape

Gel beads with a very small volume are desirable for increasing the probability of initial entrapment of a single cell (Weaver et al. 1988). However, for microcultivation, the bead should also have a size that allows space enough for the cell to divide and form a microcolony inside. For this reason, a size range of 20–80 μm was aimed at. Oil type, rotation impeller and the combination of the three factors showed to have a correlation with regard the bead size ($P < 0.01$).

Statistical post hoc comparison among the combination of the three factors studied (hydrophobic phase, presence of baffles in the beaker and rotation speed when mixing) was performed to determine the best conditions for bead size in the desired size range. It was found that in general, smaller beads are produced when RME is used as hydrophobic phase than with RPS. However, no significant difference was found between the average size of beads produced with RPS or RME-no baffles-100 rpm and the ones produced RME-no baffles-1,000 rpm ($F = 1.35$; $P = 0.25$) (Fig. 1). Nevertheless, the latter combination resulted in too

Fig. 1 Mean values of calcium alginate bead size as a function of hydrophobic phase, presence of baffles in the beaker and rotation speed when mixing. RPS rapeseed oil; RME rapeseed methyl ester. Error bars represent standard E = error of the mean after contrast analysis



small bead size range for our intended application for microcultivation ($16.27 \pm 8.26 \mu\text{m}$) thus, not considered for further morphological characterization.

Cumulative size distribution based on the relative number of calcium alginate beads measured by optical microscopy for beads produced with different oils (Fig. 3) shows that most of the beads analysed (60–80 %) are in the aimed size range 20–80 μm for beads produced with RME (100 rpm) or RPS (1,000 rpm) and no baffles. However, in both cases, a positively skewed distribution is observed and most of the alginate beads counted are between 30 and 40 μm .

Minimally 60 % of the beads produced by each combination of factors were spherical. Although, the different parameters tested by ANOVA showed no significant difference among all beads studied for alginate bead shape ($P \leq 0.05$). However, the presence of many amorphous beads or conglomerates of alginate pieces was observed by microscopy in the samples analysed (Fig. 3a (RPS)). As reported previously, lack of sphericity can be attributed to incomplete consolidation of the drops during gelation (Poncelet 2001). Previous research has also shown that this problem can be avoided by reducing the size of the particles in the calcium carbonate slurry (Quong et al. 1998). Although, our findings also suggest that a less viscous hydrophobic phase, such as RME, can facilitate the diffusion of the oil soluble acid in the drop and immediately consolidate the calcium alginate bead.

With regard to the hydrophobic phase used, the average particle size obtained from an internal gelation process is dependent on the viscosity of the hydrophobic phase employed (Price and Monshipouri 1998; Reis et al. 2006). For this reason, we tested the use of RME, as it shows a lower viscosity compared to the rapeseed oil, with a kinematic viscosity at 25 °C of 6.49 mm s^{-2} for RME and 62.63 mm s^{-2} for RPS. In other studies aiming at producing alginate beads in a microscale or even in a nanoscale (Reis et al. 2007), for solving the viscosity problem of oil employed, it has been common to add surfactants to the hydrophobic phase when mixing while producing alginate beads by internal gelation (Poncelet et al. 1992, 1999; Liu et al. 2002). Surfactants are also commonly used in the process for avoiding coalescence among emulsified droplets (Reis et al. 2006); however coalescence does not seem to be a problem in the process suggested

when using RME for production of the alginate beads. Overall, RME is relatively easy to obtain and can be considered as a safe vegetable origin hydrophobic phase and biocompatible.

Regarding the rotation impeller factor, the lower rotation speed used is favourable as lower shear force is applied on cells to be entrapped. The use of baffles in the reaction beaker has not shown to be statistically significant, but it could favour the increase of the dispersive force.

For a final selection of the optimal conditions, morphological characteristics of alginate beads produced with RME, baffle in beaker and 100 rpm of rotation speed when mixing, were compared with beads produced with RPS, no baffle and 1,000 rpm of rotation speed.

Morphological characterization of alginate microbeads

In order to select calcium alginate microbeads with optimal shape and size, the above described calcium alginate beads were examined using SEM. The SEM images showed that most of the beads were in the desirable size range (20–80 μm) (Fig. 3a), in agreement to what was also observed under optical microscopy (Fig. 4) and corresponding to the size distribution measurements (Fig. 2) and statistical analysis (Fig. 1). It was also observed that calcium alginate beads produced with RME were more spherical and with a smoother surface than the ones produced with RPS (Fig. 3a, b). A spherical shape is desired for optimal mass transfer between medium components and the entrapped cells during the cultivation of the microbeads and for that reason beads produced with RME were selected for further microcultivation tests.

The internal structure was studied for broken beads which showed quite similar features for the calcium alginate beads produced with RME and RPS (Fig. 3c). For both types of beads, gel structures were relatively uniform and exhibited large pores, which were approx. 7–8 times larger than the surface pore size range. Alginate beads formed by internal gelation have larger internal pores than those formed by the classical extrusion method (Liu et al., 2002), which is externally gelated. This occurs because during the internal gelation, there is a more homogenous distribution of alginate and calcium citrate in the aqueous droplet which will immediately form the calcium alginate

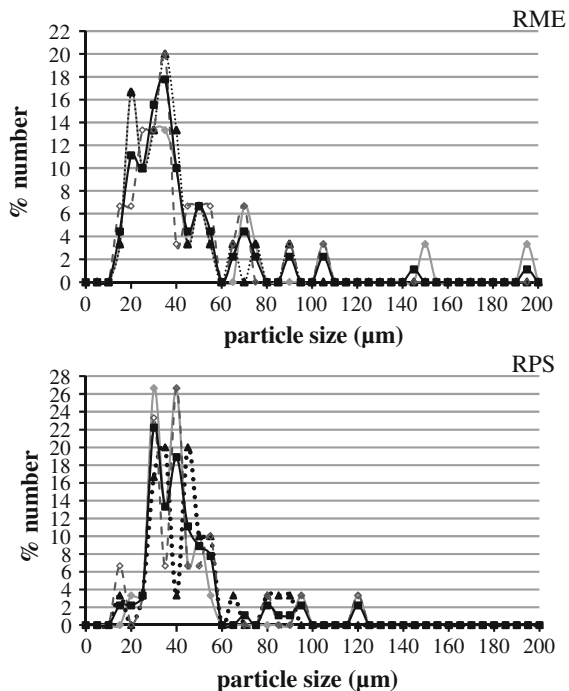


Fig. 2 Size distribution for calcium alginate beads produced with RME (RME, beaker with no baffles and 100 rpm of rotation when mixing) and RPS (beaker with no baffles and 1,000 rpm of rotation when mixing). (black diamond, black triangle, white diamond) Represents each measurement replicate, (black square) cumulative size distribution

bead when Ca^{2+} is released by the oil containing acid. In comparison, the external gelation (such as the traditional extrusion of alginate in CaCl_2 solution), where the Ca^{2+} immediately reacts with the glucuronic acid residues at the droplet surface and forms a calcium gradient as it diffuses intrinsically forming the gel (Quong et al. 1998; Liu et al. 2002; Reis et al. 2006). The large internal pores are advantageous for efficient diffusion of the cultivation medium in the Calcium alginate bead and for the microcolony expansion (Fig. 3c). The space for microcolony formation can be compared with the single cell shown on Fig. 3c, (RPS).

In contrast to the large internal pores, those on the surface of the alginate beads were reduced for both types of beads (Fig. 3d) with approx. 0.07–0.65 μm size range. It is possible that during the partitioning of alginate beads from the oil dispersion to the CaCl_2 solution, a second gelation step occurs, similar to the external gelation process, taking place on the surface and increasing its Ca^+ concentration, causing the

surface pores to decrease in size. Such behaviour would suggest that the external pores are small enough to prevent release of cells from the alginate bead during cultivation of entrapped cells, but could still allow the exchange of different compounds such as substrate, growth and signalling factors and by-products between the cells and the surrounding medium.

Entrapment of single cells and microcultivation of entrapped cells

C. sulfatirredunces was used at 8×10^6 cells ml^{-1} in a new batch of alginate beads produced with RME-no baffles-100 rpm for single cell entrapment and microcultivation studies. A slight increase in the initial cell load was applied to fit the Poisson statistics, considering that most of the alginate beads are between 30 and 40 μm .

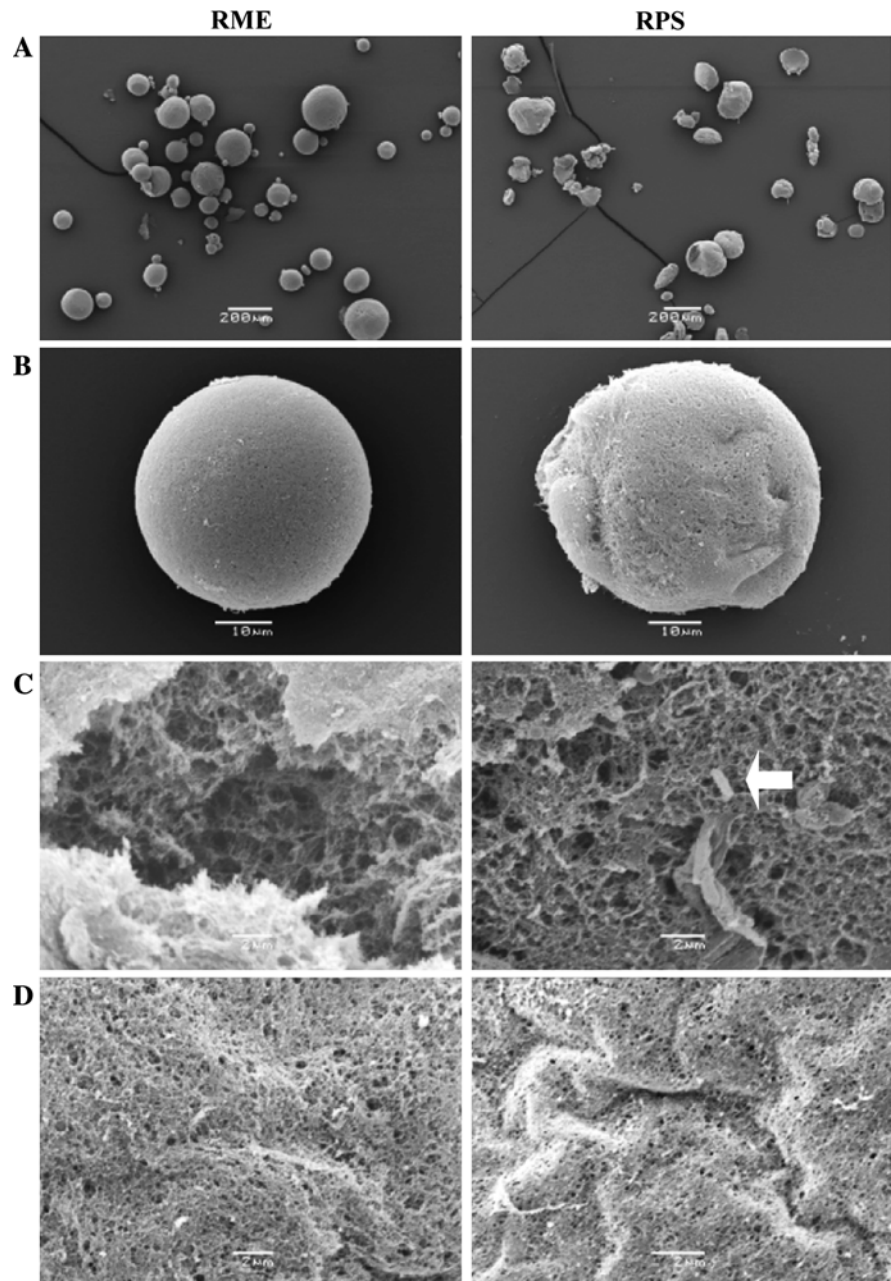
Not all the initial alginate was transformed into microbeads: some fraction remained on the walls and the bottom of the beaker thereby forming aggregates resulting in loss of yield due to the high viscosity of alginate-in-oil dispersion (Poncelet et al., 1992).

As a result, empty calcium alginate beads (Fig. 4a) and beads containing one cell (Fig. 4b, c) were observed confirming the entrapment of single cells. Fluorescence viability stain with Syto 9 (Invitrogen) also showed that cells were viable after the entrapment procedure. Considering the average volume of the microbeads and the initial cell concentration, it is possible to determine encapsulation success according to Poisson probability. Approx. 16 % of the alginate beads were occupied with bacterial cells. From the occupied beads, 94 % contained a single cell and 6 % contained up to 7 cells.

Distinguishable microcolony formation was observed in the microbeads after cultivation for 36 h with cellobiose as carbon source (Fig. 4d).

As observed previously, it is possible to use beads of natural polymers for cultivation of mammalian cells, yeast and bacteria starting from one single cell entrapped (Weaver et al. 1991; Manome et al. 2001, Zengler et al. 2002, Shiqui et al. 2012) and it offers a great potential to explore biotechnological applications for cells difficult to cultivate or slow growers by traditional techniques. It is however, the first time that alginate is used for the cultivation of single cell anaerobic bacteria in a microbead.

Fig. 3 Scanning electron microscopy of calcium alginate beads produced with RME oil encapsulating *Clostridium sulfatireducens* CCUG 50825 and produced with RPS encapsulating consortia L1. **a** Overview of the produced beads; bar 200 μm , **b** calcium alginate bead; bar 10 μm , **c** view of the porous inside the matrix, arrow indicating a bacterial cell observed in a broken bead; bar 2 μm , **d** the porous surface of calcium alginate bead; bar 2 μm

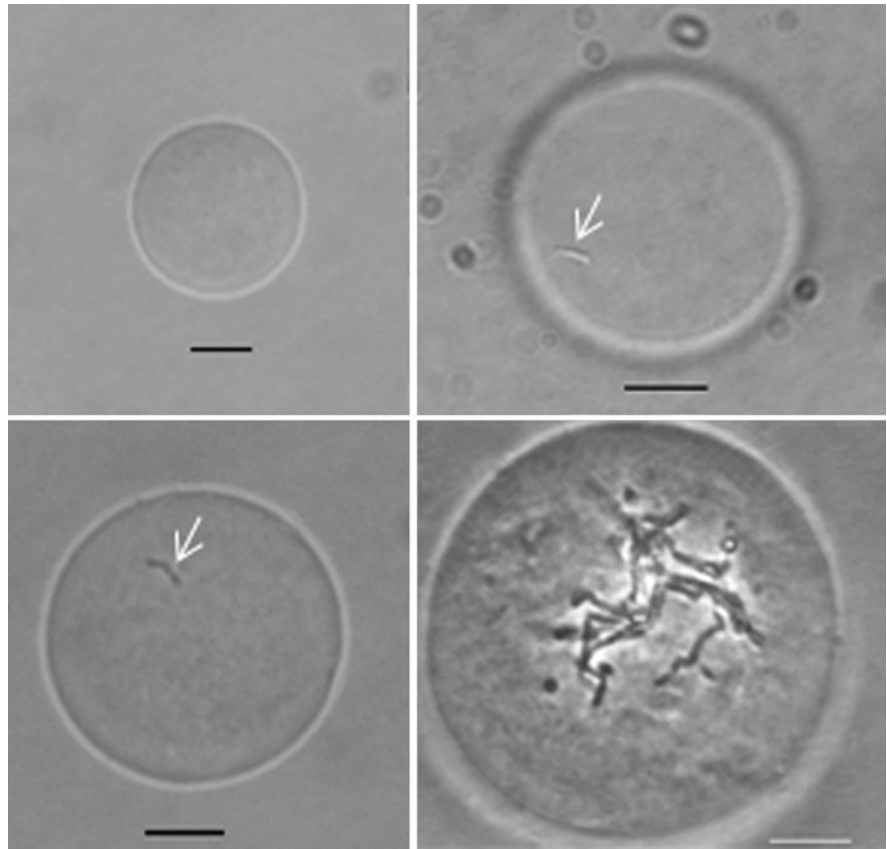


The analysis and sorting of the alginate microbead containing the microcolony can be done in future by flow cytometer equipped with a sorter system. Studies in this direction are carried out at present in our laboratory.

Finally, anaerobic organisms have to a large extent been neglected in biotechnology. Microcultivation of anaerobic microorganisms in microbead can improve isolation rate and yield and biomass

growth on this group of cells that has been considered less convenient for cultivation, compared to the aerobic ones. Furthermore, anaerobes are generally speaking growing much more slowly than what aerobes do. When the interest now increases for utilizing anaerobes, it may be important to be able to isolate also slowly growing species. The technology presented here seems to hold promises in that direction.

Fig. 4 Microscopic image of calcium alginate microbead. **a** Empty bead, **b** and **c** beads containing one single cell, arrow indicating the single cell in the bead, **d** bead containing a microcolony after 36 h cultivation. Images a to c in bright field, d in phase contrast. Bar indicates 10 μm



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