Isolation and accelerated analysis of microorganism by means of microencapsulation

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Introducction

During last years, main efforts have been involved in the selection and identification of new, rare or just previously unrecognized microorganisms looking for "selective" or "surprising" behaviours. Interest includes searching of new entities and biodiversity, discovery of metabolites with pharmaceutical activity, selection of high producing cell lines, etc. (Newman 2004). This research has been supported by great advances on analytical techniques mostly involved in identification issues. Albeit this significant evolution, initial microorganism isolation steps remain mostly the same for years, being more than a few times the main reason for failures or no microbial recovery (i.e. with minority lines, non suitable or unknown culture media, etc). The combination of a single cell encapsulation procedure and flow cytometry has aroused as an efficient process for microbial isolation that enables cells to grow with selected nutrients present at environmental conditions (Zengler 2005)

Here we present a very soft and controlled procedure that permits specific microparticle design ensuring microorganisms survival (Martin-Banderas 2005). Flow Focusing is a versatile technique that allows individual cell/microorganism encapsulation by adjusting the desired particle size, even down to 10-20 micron, including proper culture media inside the particle favouring specific growth by controlling environmental conditions. After a few hours of incubation in selected conditions, individual microorganisms inside the particles will growth selectively forming small colonies depending on particular culture and external environment. These microcolonies inside the particles will be detected using flow citometry and isolated with a cell sorter.

The Flow Focusing technology is being validated as a procedure for very smooth microorganism encapsulation in order to obtain huge amounts of different data reducing working time. In fact, due to the versatility of Flow Focusing, many parameters could be checked including type of microorganism, matrix, medium, environment, etc., providing accelerated analysis without microorganism damage (Gañan-Calvo 2001).

Results on microencapsulation of different thermoresistent mutants of yeast using Flow Focusing technology is shown.

Materials and methods

Materials

Microcapsule matrix polymer was sodium alginate from Fluka (Steinheim, Germany) and calcium chloride ($CaCl_2$ _ $2H_2O$, Riedel-de-Haën, Germany).

Yeast, thermoresistent mutants modified to produce GFP (BY4147) and thermosensitive mutants modified to produce GFP (spt6). Yeast strains were kindly donated by Department of Genetics of the University of Seville and were grown in YEPD medium.

Simple FF Nozzle(fig. 1): D_0= 200 µm, D= 200 µm, H= 180 µm. Syringe pump (Harvard), Compressed air line, Magnetic stirrer (SBS), Optical microscope (Leica DM LS), Optical and fluorescence microscope equipped with a camera (Leica DC 350F).

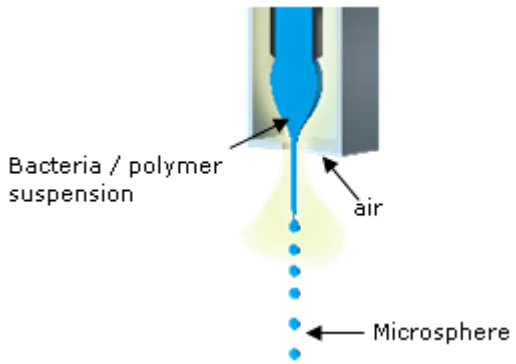

Figure 1: simple FF nozzle

Methods

Cell microencapsulation: Four types of microcapsules were preparated for this work: empty microcapsules and three more ones enclosing different type of cells, BY 4741, Spt6 and a mixture of both strains.

After brief centrifugation, the cell pellet was suspended in a mixture of 1% sodium alginate (1% w/v, 8 mL) and 20% YEPD medium (2 mL). The dilute alginate suspensions (≈ 10^6 cells/mL) were sprayed with a nozzle of **200 µm** using very **soft** conditions, 10 mL/h for the suspension and a very low pressure of air (**70 mbar**) as a focusing fluid. The droplets were collected in 3% calcium chloride solution and the emulsion was stirred for at least 15 minutes to yield microparticles of **50µm** with a very low concentration of cells inside, nearly individually encapsulated.

Microparticle characterization: Diameter and microcapsules structure were observed and photographed by using an optical microscope and an image processing program (Image J. 1.30v). Microencasulated yeasts were observed using optical and fluorescence microscopes.

Results and Discussion

Microcapsules were prepared at room temperature, under sterile conditions as it is described in previous sections. Results are summarized in table I.

Nozzle diameter	Suspension flow rate	Pressure	Microparticle size
200 µm	10 mL/h	70 mbar	**50 µm**

Table I. Technical data for microparticle encapsulation

In fig. 2-4 are shown some examples of fluorescence and optical microscope images from immobilized yeast before incubation process. As it is shown, due to the dilute suspensions that are used, there are a lot of empty particles in the samples.

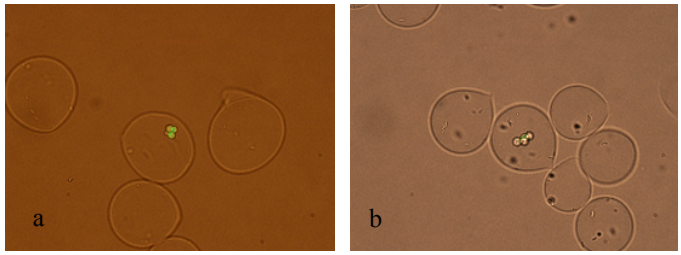

Figure 2: Examples of possible types of microparticles and microencapsulated material obtained using the FF technology: (a) AlNa/BY4147 yeast microparticle (50 µm); (b) BY4147 + spt6/AlNa microcapsules (50 µm)

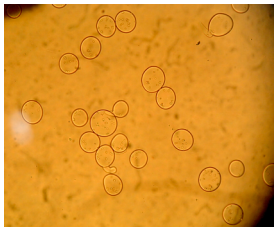

Figure 3 : Spt6 + BY4147 microparticles

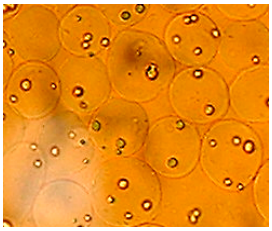

Figure 4 : Spt6 + BY4147 microparticles

The particles were incubated at 30ºC and 37ºC in a 5% CO_2 humidified atmosphere during 24 h. After the incubation period, the proliferation rate of encapsulated cells was observed by optical and fluorescence microscopy. In one hand, for microcapsules with BY 4741 (thermoresistent) we observed a high grade of proliferation in both temperatures of incubation (Fig. 5,7). In the other hand, in the case of microcapsules with Spt6 (thermosensitive), the growth was only observed at 30ºC (Fig, 6,8).

Particles where both strains have been encapsulated showed different behaviour at both temperatures. As it was expected, at 30 ºC all the particles showed yeast growth, but at 37 ºC only particles encapsulating the thermoresistent yeast showed some growth, allowing yeast strain differentiation.

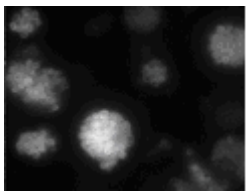

Figure 5: BY4147 microparticles at 30 ºC

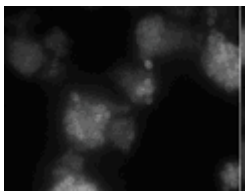

Figure 6: Spt6 microparticles at 30 ºC

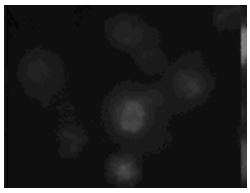

Figure 7: BY4147 micropaticles at 37 ºC

Figure 8: Spt6 micropaticles at 37 ºC

Conclusions

We have shown Flow Focusing as a very versatile and soft technology that allows cell microencapsulation in very small microparticles (50 μm) ensuring microorganism viability.

Using diluted cell suspensions, individualize microencapsulation could be controlled, allowing selection of biodiversity.

As an example, different yeasts microencapsulation have been shown. The viability of immobilized yeast in sodium alginate microcapsules was verified under different growing conditions (30 ºC y 37ºC). In every condition assayed, thermoresistant BY4147 yeast growth and GFP expression took place. In the other hand, thermosensitive Spt6 yeast only grows under temperatures below 30 ºC. Results shown in figures 5-8 indicate the possibility to induce selective growth controlling environmental conditions.

References

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