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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Effect of various parameters on viability and growth of bacteria immobilized in sol–gel-derived silica matrices

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Abstract Immobilized bacteria are being extensively used for metabolite production, biocatalysts, and biosensor construction. However, long-term viability and metabolic activity of entrapped bacteria is affected by several conditions such as their physiological state, the presence of high-osmolarity environments, porous structure and shrinkage of the matrix. The aim of this work was to evaluate the effect of various parameters on bacteria immobilized in sol–gel-derived silica matrices. With this purpose, we evaluated the stress of immobilization over bacteria cultures obtained from different growing states, the effect of cell density and bacteria capability to proliferate inside matrices. Best results to attain longer preservation times were obtained when we immobilized suspensions with an optimized bacterial number of 1×10^7 cfu/gel in the presence of LB medium using aqueous silica precursors. Furthermore, the impact of osmotic stress with the subsequent intracellular trehalose accumulation and the addition of osmolites were investigated. Shorter preservation times were found for bacteria immobilized in the presence of osmolites while trehalose accumulation in stressed cells did not produce changes on entrapped bacteria viability. Finally, nutrient addition in silica matrices was studied indicating that the presence of a carbon source without the simultaneous addition of nitrogen was detrimental for immobilized *E. coli*. However, when both carbon and nitrogen sources were present, bacteria were able to survive longer periods of time.

Keywords Additives · Bacteria · *Escherichia coli* · Immobilization · Living cells · Sol–gel

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Introduction

Bacteria are being extensively used for biocatalysis, biosensors, and bioremediation because they offer the possibility to work in environmentally friendly conditions. In many cases, living cells cannot be used as such and need to be stabilized via encapsulation in suitable host matrices (Livage and Coradin 2006). Their immobilization in silicate matrices provides advantages over free cells such as: increased metabolic activity, protection from environmental stresses and toxicity, increased plasmid stability and they may act as cell reservoir systems prolonging reaction times (Bottcher et al. 2004; Desimone et al. 2006). However, bacteria immobilized in silica matrices are exposed to many types of stresses like excess of osmotic pressure due to sodium ions present in some aqueous silicate precursors (Nassif et al. 2003), stress due to gel formation or the drying process which causes shrinkage of the matrix and damage of entrapped cells. Stewart and Robertson (1989) suggested that entrapped growing bacteria can exert a substantial stress on their surroundings. Sun et al. (2007) reported an increase in the intracellular trehalose and glycerol content and superoxide dismutase activity of cells entrapped in liquid core microcapsules of alginate–chitosan.

Trehalose and glycerol are examples of compatible solutes which are accumulated in cells under hyperosmotic conditions to allow them tolerate osmotic stresses (Oren

2003). Trehalose has demonstrated to decrease oxidative damage caused by oxygen radicals (Benaroudj et al. 2001) and to accumulate in response to heat shock and cold exposure, allowing bacteria viability at low temperatures (Kandror et al. 2002). It was also shown that trehalose considerably increase the tolerance of *Escherichia coli* to drying processes, whether added as an excipient prior to drying or accumulated as a compatible solute in response to osmotic stress (Welsh and Herbert 1999). Nevertheless, few studies have been made to show trehalose protective effect over entrapped bacteria, and most of them focus in the exposure to drying conditions of immobilized bacteria and no in the stress of immobilization itself (Champagne and Gardner 2001; Tessema et al. 2006).

Some other additives have been tested for bacteria protection during encapsulation. According to Nassif et al. (2002, 2003), the main requirements for such additives include water solubility, biocompatibility and maintenance of silica gel cohesion. Best results, after 1 month, were obtained with glycerol, a well-known cryoprotective agent currently used to preserve bacteria during lyophilization. The presence of polyols can also modify pore volumes, surface area, and state transitions of water and solid components. These are important aspects to take into account in the formulation of the encapsulating matrices to preserve biological stability (Desimone et al. 2008). Despite the incorporation of humidity-storage compounds and nutrients in silica matrices may improve the bioactivity of entrapped cells, there have been no systematic investigations on the effect of such co-immobilized additives (Bottcher et al. 2004).

Therefore, the aim of this work was to evaluate the effect of osmotic stress and the presence of compatible solutes, including glycerol, mannitol, and trehalose, on immobilized bacteria performance. The addition of nutrients inside the matrices was also evaluated. Cell capability to proliferate in a confined porous space was assessed when nutrients were incorporated into silica gels. Other evaluated parameters included the stress of immobilization over bacteria cultures obtained from different growing states and the effect of cell density on gel formation and bacteria viability.

Furthermore, as reported in a previous work (Alvarez et al. 2007), the effect of citric acid as a gelation agent was compared to the use of inorganic acids on bacteria viability preserved for 550 days.

Materials and methods

Chemicals

Silicon dioxide (SiO_2) and tetraethoxysilane (TEOS) were purchased from Fluka (Buchs, Switzerland). Sodium silicate was obtained from Riedel de Haën (Seelze, Germany). Luria–Bertani broth (LB) media components and agar used for bacterial plate counts and cultures were purchased from Difco (Kansas, MO). Trehalase (EC 3.2.1.28) was obtained from Sigma (St. Louis, MO) and glucose oxidase assay kit was provided by Wiener Lab (Rosario, Argentina). All other reagents were of analytical grade and were used as received without further purification.

Bacterial strains, culture conditions, and viability determination

E. coli (ATCC #8739) was kindly provided by the Microbial Culture Collection of the Facultad de Farmacia y Bioquímica (CCM 29), Universidad de Buenos Aires. An *E. coli* strain from glycerol stock was plated on LB agar and incubated for 24 h at 37°C. Selected colonies were cultured in LB medium, up to OD (600 nm) 0.800, centrifuged and resuspended in LB medium. The number of colony-forming units (cfu) per milliliter of this suspension was determined by the plate count technique before its utilization in immobilization studies.

E. coli cells obtained from cultures either in exponential phase (12 h), stationary phase (24 h), or from colonies formed in LB agar were resuspended in phosphate buffer and immobilized in sodium-silicate-derived matrices in order to investigate the influence of bacteria growing state on the viability after immobilization. In order to find the optimal number of bacterial cells to be immobilized, cultures in stationary phase were resuspended in phosphate buffer and adequate decimal dilutions were performed to obtain bacterial suspensions ranging from 10^3 to 10^{12} cfu for their immobilization in sodium silicate gels. A similar assay was performed in LB medium with bacterial suspensions ranging from 10^2 to 10^{11} cfu.

For viability determination, decimal dilutions in 0.9% *w*/*v* NaCl of the free bacteria suspension were plated in triplicate in LB agar and incubated at 37°C for 48 h. For immobilized cell count, each gel matrix (about 200 mg) was disrupted by using a sterilized glass rod, homogenized in 0.5 ml 0.9% *w*/*v* NaCl and used for viable cell determination, as done with the bacterial suspension. All determinations were made in triplicate and statistically analyzed by one-way ANOVA.

Immobilization of bacteria in sol–gel-derived silica matrices

The procedure was performed as previously reported (Alvarez et al. 2007). Basically, 1 g of SiO_2 was mixed with 4 ml of 2 M NaOH and incubated at 80°C until complete colloidal suspension was attained. For sodium

silicate, 1 g of sodium silicate was mixed with 6 ml water and then incubated at 80°C until complete colloidal suspension was attained. Each sol (colloidal suspension) was allowed to reach room temperature and acidified to pH 6.50 with 0.75 M citric acid or 1 N HCl. Afterwards, 0.15 ml of a bacterial suspension in 0.2 M phosphate buffer pH 7.00 were mixed with an equal volume of the above described colloidal suspension.

When tetraethoxysilane (TEOS) was used, the sol was prepared as previously reported (Desimone et al. 2002), by sonicating (Transsonic 540 sonicator, 35 kHz; Elma, Singen, Germany) a mixture of 1 ml TEOS, 0.2 ml water and 0.06 ml 0.05 M HCl for 30 min at 20°C. After addition of 2 ml water, excess ethanol was eliminated under N_2. A bacterial suspension (0.15 ml) obtained in 0.2 M sodium phosphate buffer pH 7.00, was mixed with an equal volume of the sol solution. The solutions were left for 2 min until gelation. Storage stability in inorganic matrices was studied at 4 and 20°C.

Effect of osmotic stress: intracellular trehalose accumulation

Three 250-ml Erlenmeyer flasks with LB medium were prepared with 0.1, 0.3, and 0.6 M NaCl final concentration. Each flask was inoculated with *E. coli* and cultured for 24 h. They were afterwards centrifuged and cells resuspended in 0.2 M sodium phosphate buffer pH 7.00. These suspensions were used for bacteria immobilization in TEOS matrices in order to expose bacteria to a stressful situation. Immobilized bacteria viability was evaluated as a function of time.

Culture samples of 50 ml, from flasks supplemented with 0.1, 0.3, and 0.6 M NaCl, were harvested by centrifugation for 5 min. Cell pellets were washed with 10 ml of isotonic saline solution and pelleted by centrifugation for five additional minutes. The supernatant was discarded and cells were resuspended in distilled water and disrupted by sonication. Then, supernatant proteins were precipitated with 500 mM trichloroacetic acid, centrifuged, and the supernatant used to determine intracellular trehalose and total sugar content. Trehalose in the supernatant was converted to glucose with trehalase and was then measured by a glucose oxidase assay kit. The pre-existing glucose was determined in a reaction without trehalase and subtracted from total glucose. Total sugar in the supernatant was measured by the Anthrone method (Lillie and Pringle 1980). Cell pellets were used to determine mass dry weight.

Effect of osmolites over entrapped bacteria

LB medium was prepared with 0.1 M NaCl, inoculated with *E. coli* and cultured for 24 h. It was afterwards centrifuged and cells resuspended in 0.2 M sodium phosphate buffer pH 7.00. This suspension was fractioned and supplemented with different compatible solutes such as 10% glycerol, 10% mannitol, 2.5 and 10% trehalose. All of them were used for bacteria immobilization in TEOS-derived matrices as indicated before. Viability for 550 days was evaluated for immobilized bacteria with different osmolites.

Influence of co-immobilized nutrients on encapsulated bacteria survival

LB medium was prepared with 0.1 M NaCl, inoculated with *E. coli* and cultured for 24 h. It was afterwards centrifuged and cells resuspended in 0.2 M sodium phosphate buffer pH 7.00. This suspension was fractioned and supplemented with LB medium, 2 % glucose, 2% glucose, and 0.5 % $(NH_4)_2SO_4$ or without any added amendments. They were used for bacteria immobilization in TEOS and silicate-derived matrices as indicated before. Viability after 45 days was evaluated for immobilized bacteria.

Results

Effect of bacteria growing state and cell density

Bacteria from three different growing states were immobilized in citric acid sodium silicate-derived matrices without nutrients. As shown in Table 1, after 10 days, no differences were found for immobilized bacteria viability obtained from cultures in exponential, stationary phase, or when single colonies grown in LB agar were resuspended in phosphate buffer.

Different bacterial suspensions ranging from 10^2 to 10^{12} cfu were immobilized in sodium silicate. Figures 1 and 2 show viability after 2 days of immobilized *E. coli*, resuspended in phosphate buffer and in LB medium prior to encapsulation respectively. As it can be seen in Fig. 1, the number of entrapped bacteria was similar to the number of cells contained in the initial suspension for low-density

Table 1 Effect of different growing conditions over bacteria immobilization

Growing condition	Initial (cfu)	10 days (cfu)
Exponential phase	3.02×10^8	4.10×10^7
Stationary phase	2.70×10^8	4.28×10^7
Grown in LB agar	3.00×10^8	4.80×10^7

Mean value from triplicated samples. Analysis of variance (ANOVA) shows no significant differences in the number of viable bacteria found 10 days post-immobilization with a level of significance at 0.05

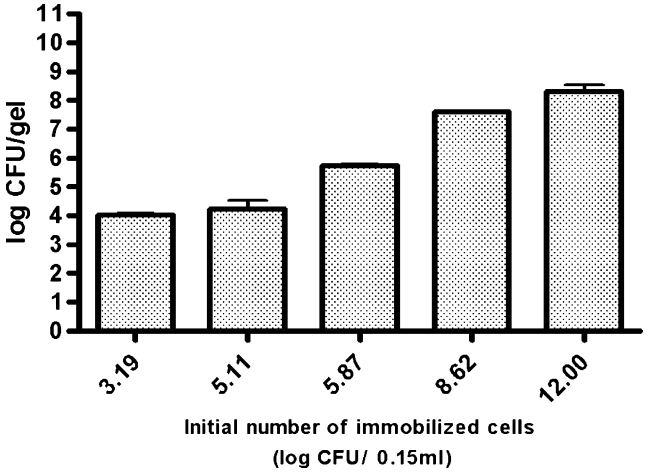

Fig. 1 Number of entrapped cells from different bacteria suspensions in phosphate buffer 2 days after immobilization

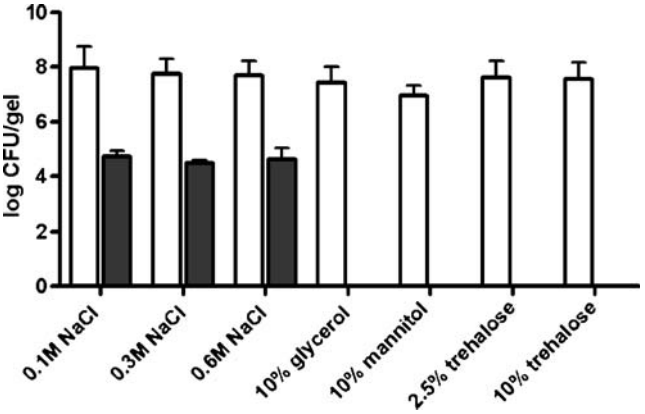

Fig. 3 Effect of osmotic stress and osmolite addition over entrapped bacteria after (*empty square*) 10 days and (*filled square*) 550 days post-immobilization

suspensions (below 10^9 cfu). Nevertheless, when high cell density suspensions were used (10^{12} cfu), the number of countable bacteria did not exceed 10^9 cfu per gel. In Fig. 2, a similar experiment was conducted but this time using an LB suspension to allow bacteria growth inside gels. The number of immobilized bacteria reached a mean around 10^7 cfu per gel for every initial suspension under study.

Effect of osmotic stress and osmolite addition on entrapped bacteria viability

As it has been reported in previous studies (Coiffier et al. 2001; Desimone et al. 2005), alkoxide precursors are not as biocompatible as sodium silicate because they do not follow an aqueous route of polymerization. Moreover, silicon alkoxide precursors are usually not fully hydrolyzed during the first acid-catalyzed step and alcohol can still be released during condensation and aging in the presence of the immobilized bacteria. Therefore, we have chosen TEOS-derived matrices in order to expose entrapped bacteria to a higher level of stress. As shown in Fig. 3, 10 days after immobilization in TEOS-derived gels, the number of countable *E. coli* was not significantly different (p=0.7430) among matrices with additives such as glycerol, mannitol, and trehalose or in those without any additives where immobilized bacteria had previously been exposed to various NaCl concentrations.

On the other hand, 45 days after immobilization significant differences in bacterial count were found. There were no countable bacteria in TEOS-derived matrices where glycerol, mannitol, or trehalose was added. In gels where bacteria were exposed to various NaCl concentrations prior to encapsulation, a high bacterial count was found until 550 days when approximately 10^5 cfu were found.

Intracellular trehalose levels were determined in *E. coli* cultures exposed to 0.1, 0.3, and 0.6 M NaCl, respectively,

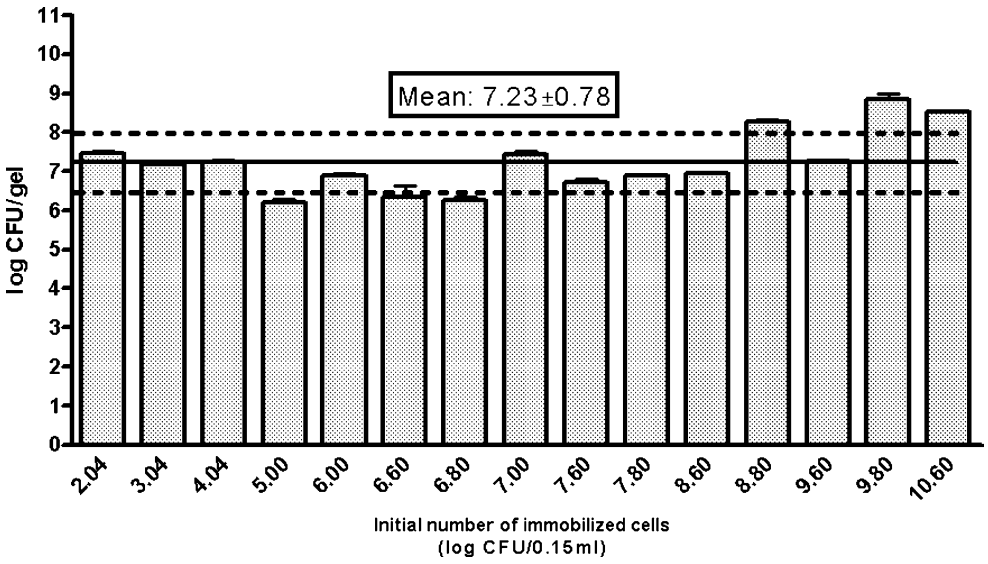

Fig. 2 Number of entrapped cells from different bacteria suspensions in phosphate buffer amended with LB medium 2 days after immobilization

which were afterwards used for bacteria immobilization. According to Fig. 4, trehalose was only accumulated in high levels (three times higher than in the other conditions) when exposed to 0.6 M NaCl. Cells exposed to 0.3 M NaCl had a similar trehalose content than those grown in 0.1 M NaCl.

Even though intracellular trehalose levels were higher for bacteria grown in 0.6 M NaCl, the number of culturable bacteria after 550 days of immobilization was not significantly different (p=0.5304) among the three NaCl conditions. Furthermore, encapsulated bacteria in gels containing compatible solutes were also grown in a culture media with 0.1 M NaCl but did not remain culturable after 550 days.

Influence of co-immobilized nutrients on encapsulated bacteria survival

For this purpose, *E. coli* were suspended in phosphate buffer and immobilized in two different silica precursor, TEOS or sodium silicate, with no additives and in the presence of nutrients such as LB medium, glucose, and glucose together with ammonium sulfate as carbon and nitrogen sources, respectively.

As shown in Fig. 5, bacteria viability during 45 days was not altered in tubes where no nutrients were added and in tubes with LB medium or glucose and ammonia. For tubes containing only glucose, the number of countable bacteria drastically decreased for both TEOS and (citric acid) sodium silicate-derived gels and, after 45 days, no viable bacteria were found. Glucose levels were analyzed in those gels where no countable bacteria were found. It was observed that glucose levels had diminished (Fig. 6) as well as bacteria viability, showing that encapsulated bacteria are not in a resting state but metabolically active and in the presence of a carbon source (glucose, trehalose, glycerol, or mannitol) but under nitrogen starvation, rapidly died.

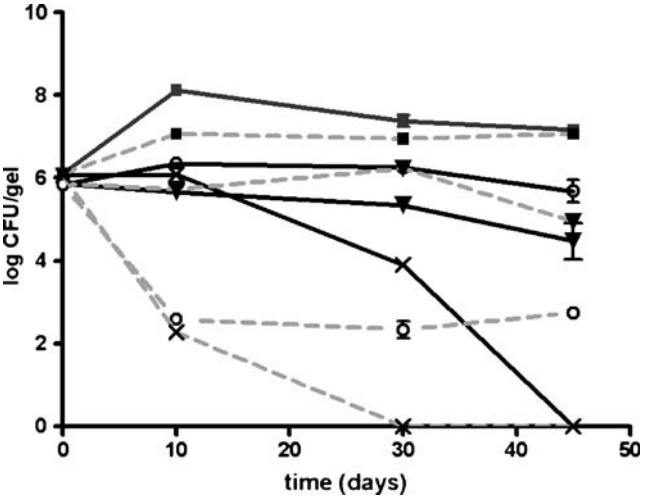

Fig. 5 Effect of nutrients over bacteria viability immobilized in silicate (*complete line*) and TEOS (*dashed line*) matrices. Gels with (*filled square*) LB medium, (*ex symbol*) glucose, (*empty circle*) glucose and ammonia or (*filled inverse triangle*) with no amendments

In order to rule out the possibility that media acidification, after carbon-source consumption, might be causing bacteria death, we measured the internal matrix pH with an ISFET (Ion Sensitive Field Effect Transistor) and in all cases we found an approximate value of 6.50.

Effect of organic acids on entrapped bacteria viability

In a previous manuscript, we have reported the beneficial use of citric acid instead of hydrochloric acid in the gelation

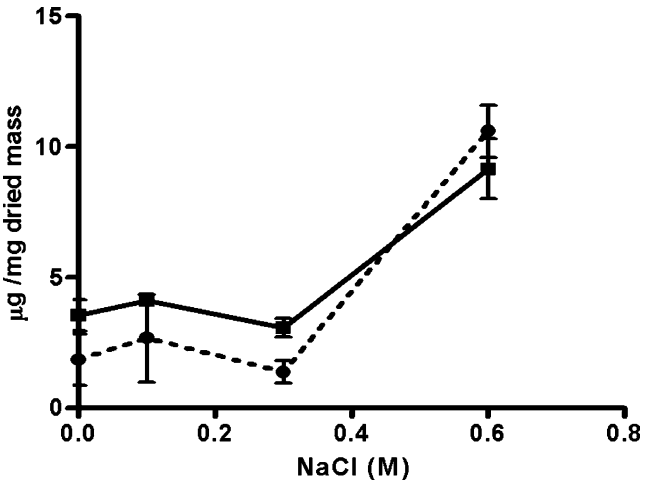

Fig. 4 Trehalose (*dashed line*) and total sugar (*complete line*) intracellular content when *E. coli* cultures were exposed to 0.1, 0.3, and 0.6 M NaCl

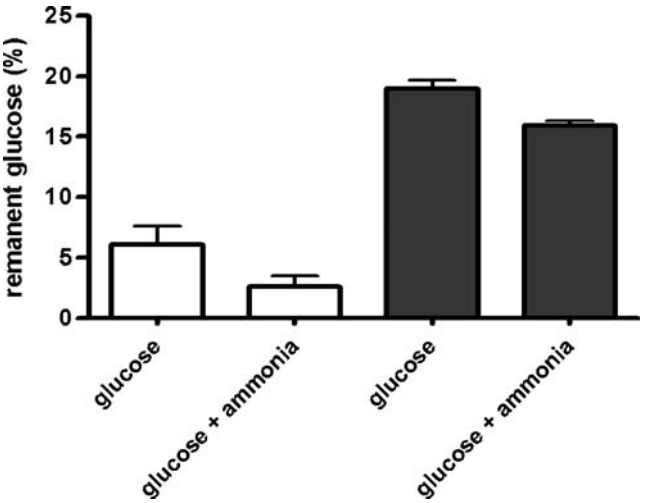

Fig. 6 Relative remaining percentage of glucose in (*empty square*) silicate and (*filled square*) TEOS-derived matrices with immobilized *E. coli* after 45 days

process for immobilized bacteria during a 90-day period. After 550 days, we have followed bacteria viability in silicate matrices obtained from two different precursors (sodium silicate and SiO_2) and found even more remarkable differences between acids. As shown in Fig. 7, when gels were stored at 4°C, *E. coli* viability was approximately two orders of magnitude higher in citric acid gels than in those gels where hydrochloric acid was used. When gels were stored at 20°C, the difference between acids was inferior but still significantly different (p=0.0131). In this assay, TEOS matrices were not studied because they confer immobilized bacteria a more stressful environment. Moreover, we immobilized a suspension with an optimized bacterial number (according to our first experiment) in the presence of LB medium to attain longer preservation periods of time. No differences were observed in bacteria viability for silicate matrices obtained either from sodium silicate or SiO_2 aqueous precursors.

Discussion

Effect of bacteria growing state and cell density

It is known that after nutrients are consumed from the medium and growth-suppressing factors accumulate, bacteria leave the exponential phase and enter a stationary state characterized by cell resistance to a variety of environmental stresses. The σ^S subunit, encoded by rpoS of RNA polymerase, seems to be involved in the cessation of growth and in the acquisition of resistance (Kabir et al. 2004). For this reason, we expected bacteria in stationary phase to face immobilization stress in better conditions. However, no differences were found when bacteria in different states of growth were immobilized; indicating that bacteria culture conditions did not have a profound effect on bacteria immobilization stress.

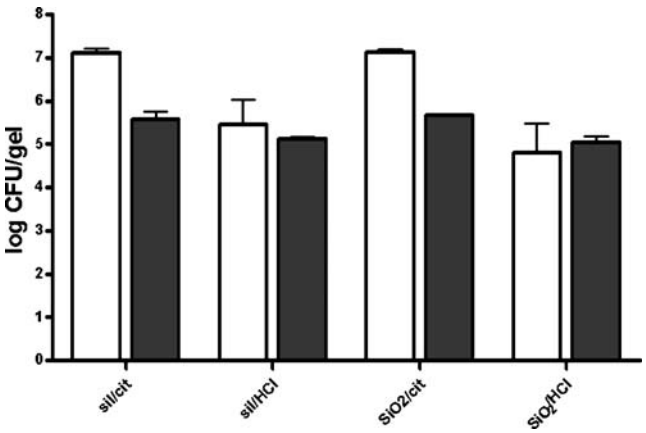

Fig. 7 Effect of organic or inorganic acids on viability of entrapped bacteria after 550 days stored at (*empty square*) 4°C or (*filled square*) 20°C

When we evaluated the number of *E. coli* cells to be entrapped in the absence of nutrients, we found that for bacterial suspensions below 10^9 cfu, the number of cells that remained countable inside the matrix was similar to the number of cells initially added in the immobilization process. On the other hand, when bacterial suspensions above 10^9 cfu were used, the number of cells immobilized in the silica matrices was lower than the number of bacteria in the suspension, showing the possibility that gels might have a maximum capacity to host bacteria. Moreover, if the assay was performed in the presence of nutrients and low-density suspensions were used, immobilized bacteria had the chance to replicate inside the matrices and reach higher populations, with a media bacterial count around 10^7 cfu per gel.

Effect of osmotic stress and osmolite addition on entrapped bacteria viability

In regard to the use of compatible solutes as additives, it was reported that glycerol had a beneficial effect on encapsulated bacteria during the first 30 days of entrapment (Nassif et al. 2002, 2003). This additive is located around bacteria, acting as a physical barrier that avoids direct contact between the silica gel pore surface and the bacteria (Ferrer et al. 2006). However, in long storage periods, other effects should arise. Herein, negative results on bacteria viability were found when any of the compatible solutes tested in this work were added, indicating that not only structural parameters were involved. Exogenous trehalose as well as glycerol and mannitol produced bacterial death after a 550-day storage period, while bacteria entrapped in gels with no additives were still viable in such a period of time. Glycerol and mannitol could be used for *E. coli* as carbon and energy sources (Hempfling and Mainzer 1975; Murarka et al. 2007). Trehalose can also serve as the sole source of carbon and energy as *E. coli* has a periplasmic trehalase that degrades trehalose to glucose (Styrvold and Strom 1991). Therefore, they may maintain immobilized bacteria in an active metabolic state which could provoke bacterial death during storage time.

On the other hand, osmotic-induced bacteria (grown in 0.6 M NaCl) which accumulated internal trehalose in high level, were viable after 550 days in contrast to bacteria with exogenous trehalose supplies. According to Kandror et al. (2002), in most cases, these organic compatible solutes are not metabolized by the cells that accumulate them but play a major role in cell protection. Even though bacteria exposed to lower NaCl concentrations (0.1 and 0.3 M) did not accumulate high levels of intracellular trehalose, still, they were viable after 550 days. This evidence suggested

that trehalose intracellular accumulation was not responsible for bacteria survival in such a long period of time, but there was a detrimental effect of the additives mentioned before over long-term encapsulated bacteria.

Influence of co-immobilized nutrients on encapsulated bacteria survival

When a complete LB media was incorporated into silicate gels, entrapped bacteria were viable and in high numbers after 550 days. Such effect was also observed when glucose and ammonia were added together as nutrients, which could suggest that the presence of nutrients is not a deleterious factor for immobilized microorganisms as long as both carbon and nitrogen sources are present. This is supported by the fact that when cells are deprived of ammonia, they attempt to scavenge nitrogen from alternative sources by activation of certain genes (Kabir et al. 2004) and when this is not possible, a general shut-down of metabolism could occur. *E. coli* is not able to replicate in the absence of environmental nitrogen unlike *S. cerevisiae* which may use internal nitrogen stores like biopolymers, presumably primarily protein (Brauer et al. 2006).

Effect of organic acids on entrapped bacteria viability

Finally, the use of organic acids like citric acid instead of inorganic ones as gelation agents, has been found to be the best option for bacteria immobilization and preservation in long periods of time in concordance with our previous work (Alvarez et al. 2007) and for both temperatures tested (4°C and 20°C). Citric acid can not be used as the sole carbon source in the *E. coli*-particular case as shown by characteristic biochemical tests used for bacteria identification. However, it might interfere in bacteria physiological condition by inducing the synthesis of compatible solutes like glycerol (Lawrence et al. 2004) or it just might affect the physical characteristics of the matrix in terms of polymerization and porosity (Naskar 2005). Furthermore, immobilization in silica matrices using citric acid, to neutralize the alkalinity of the silica precursors, makes the technique not only biocompatible but also easier to perform, since polymerization does not occur immediately as it does when hydrochloric acid is used (Alvarez et al. 2007).

This work is, to our knowledge, a systematic analysis of the effect of various parameters on bacteria immobilized in sol–gel-derived silica matrices and should provide a better understanding on how to preserve viable bacteria in silica matrices for its possible application in the development of biosensors or bioreactors.

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