P-034 **Effect of storage conditions on stability of free and encapsulated – in plain or cysteine-supplemented alginate, *Lactobacillus acidophilus* Ki**

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INTRODUCTION AND OBJECTIVES

Lately, many functional food products have been widely released. Functional foods are foods which promote health beyond providing basic nutrition (Sanders, 1998). Probiotic bacteria are currently used in the development of functional food products (Pimentel-González, 2009; Siró et al., 2008), yet sometimes face technological challenges when incorporated in food matrices with more aggressive environments – salt, acid or oxygen concentrations. In order to be effective, the suggested concentration for probiotic bacteria is 10^6 CFU/g of a product (Shah, 2000). Encapsulation is an efficient technique to overcome such difficulties since microcapsules help in their protection from both the product intrinsic properties and the gastrointestinal tract conditions. Besides the challenges originated by the food matrices and the gastrointestinal tract there is also the challenge of conservation of the viable microorganisms throughout production and storage of the delivery food product which is also a reason for encapsulating probiotics (Allan-Wojtas et al., 2008) since the encapsulation technique can also help to protect the probiotics from the storage conditions of the product and thus increase its shelf-life in which the microorganisms are inside their effectiveness range. In this research work stability of calcium alginate capsules, produced by extrusion by aerodynamically assisted flow, throughout six months storage was studied. The effect of four storage temperatures (21, 4, -20 and -80 ºC) upon the viability of free and encapsulated cells of *Lactobacillus acidophilus* Ki was assessed. Two types of microcapsules were produced – one with calcium alginate and another where L-cysteine·HCl was utilized as a supplement to the calcium alginate matrix.

MATERIALS AND METHODS

Strain

The strain used was a commercial probiotic strain — *Lactobacillus acidophilus* Ki, previously isolated from fermented milk, obtained from CSK (The Netherlands), as ultrafrozen concentrate.

Culture preparation

The aforementioned microorganism was reactivated, and pre-culture was prepared in de Man-Rogosa-Sharpe (MRS; Biokar Diagnostics, France) broth and incubated overnight at 37 ºC. MRS was supplemented with filter-sterilized 0.05 %(w/v) of L-cysteine·HCl (Fluka, Switzerland) to lower the redox potential, and incubated in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system from Oxoid, England), to achieve anaerobic conditions. The culture was then propagated by inoculating fresh media at 10 %(v/v) and incubating it in appropriate conditions. The resulting culture was centrifuged at 4.000 rpm for 20 min, at 4 ºC. The supernatant was discarded and the pellet was ressuspended in 10 % of the original volume in a sodium chloride (NaCl; Panreac, Spain) solution at 0.85 %(w/v).

Microencapsulation procedure

The probiotic suspension was added at 10 %(v/v) to a 2 %(w/v) sodium alginate and to a 2 %(w/v) sodium alginate supplemented with 0.05 %(w/v) of L-cysteine·HCl solutions. The alginate-culture mixtures (50 mL) were then extruded using a Nisco Var J30 (Nisco Engineering AG, Switzerland) microencapsulation unit with a 0.5 mm orifice and a nitrogen pressure of 0.4 barg. The extrusion rate was 4.0 mL/min. The flow rate was controlled using a syringe pump (Genie Plus). The mixtures were extruded into 200 mL of $CaCl_2$ solution 4 %(w/v), stirred at 200 rpm. The resulting microcapsules were left in contact with the $CaCl_2$ solution for 30 min at room temperature to ensure complete solidification. Afterwards the $CaCl_2$ solution was removed through decantation and the microcapsules were suspended in Ringer (Oxoid) solution. The microcapsules were then recovered (separated from the Ringer solution) by gravity filtration, using a glass filter funnel (porosity 1; Schott Duran®, Germany).

Stability throughout storage (evaluation of storage temperature effect on cell viability)

For evaluating the effect of storage temperature on cell viability throughout six months storage, four different temperatures were chosen (21, 4, -20 and -80 ºC). At 21 ºC we maintained water activity at 0.11 (a_w = 0.11) by having a saturated Lithium Chloride (LiCl; Merck) solution inside the recipient where the samples were being stored. The free cells were the probiotic suspension used in the encapsulation procedure, which the remaining volume was afterwards distributed for sterile eppendorfs, in duplicate for each sampling time, which was then stored under the experiment conditions. The microcapsules were weighed into 25 mL sterile tubes and suspended in Ringer solution in a 1:9 (g/mL) ratio, in duplicate for each sampling time, which were then stored under the experiment conditions. Samples were taken at 0, 7, 14, 21, 31, 45, 60, 90, 120, 150 and 180 days. When two consecutive sampling times showed no viable cells for both types of capsules no further sampling was performed.

Microorganism enumeration

For the enumeration of free cells, in colony forming units (CFU)/mL, decimal dilutions – using aqueous 0.1 %(w/v) peptone (Sigma-Aldrich) and 0.85 %(w/v) NaCl, were plated on MRS agar (Biokar Diagnostics) (supplemented with filter-sterilized 0.05 %(w/v) of L-cysteine·HCl, in duplicate, and the microorganisms were enumerated according to Miles and Misra (1938), following appropriate incubation. For the enumeration of the encapsulated bacteria, in CFU/g of microcapsules, microcapsules were (in duplicate) suspended in a sodium citrate (Sigma-Aldrich, USA) solution at 2 %(w/v) in a 1:9 (g/mL) ratio and subjected to stomacher at 260 rpm for 10 min, in order to break the microcapsules. The resulting solution was then treated like the free cells, according to the protocol aforementioned.

RESULTS AND DISCUSSION

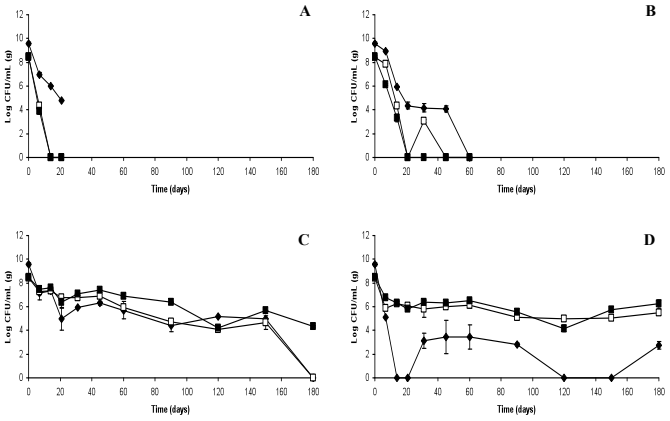

Figure 1 : Viability of *L. acidophilus* Ki free cells (♦) and encapsulated, in alginate (□) and alginate supplemented with L-cysteine·HCl (■), during six months storage at 21 (A), 4 (B), -20 (C) and -80 ºC (D).

Results at 21 ºC (Fig. 1-A) showed that after 14 days no viable cells remained inside both capsules although the free cells still presented a viable cells number of 6.02 Log CFU/mL. At 4 ºC (Fig. 1-B) encapsulation also did not provide protection to the probiotic bacteria. The encapsulated bacteria only survived for 14 days differences were observed between the capsules with and without L-cysteine·HCl. Capsules without L-cysteine·HCl presented better results. At this point the free cells viable cells number was 4.36 Log CFU/mL. After 60 days both encapsulated and free cells presented no viable cells. When stored at -20 ºC (Fig. 1-C), encapsulation conferred some protection, especially between 150 and 180 days. At 180 days, in free and encapsulated in alginate bacteria no viable cells were found and bacteria encapsulated in alginate with L-cysteine·HCl presented 4.33 Log CFU/g of viable cells. When stored at -80 ºC (Fig. 1-D) a protective effect provided by the encapsulation was observed. Throughout the storage encapsulated bacteria had superior viable numbers than free cells and, at 180 days, the difference was about 2.5 log cycles. Similar values were obtained for both types of capsules.

CONCLUSIONS

The microcapsules used in this research work revealed a protective effect upon cell viability only when stored at negative storage temperatures. When stored at -80 ºC encapsulation revealed a protective effect upon viability and the presence of L-cysteine·HCl in the alginate matrix did not improve the protective effect. When stored at -20 ºC only cysteine-supplemented alginate protected cell viability. When stored at 21 and 4 ºC encapsulation did not reveal a protective effect upon cell viability.

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