Alginate microspheres of *Bacillus subtilis*

Microesferas de alginato con Bacillus subtilis

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RESUMEN

La microencapsulación de organismos ha sido considerada como una alternativa de inmovilización de células, a fin de que éstas puedan ejercer sus funciones en forma gradual. El objetivo del presente estudio fue elaborar microesferas de *Bacillus subtilis* ya sea en forma esporulada como vegetativa.
Microesferas de *Bacillus subtilis* son preparadas utilizando alginato de sodio. Algunas propiedades típicas del sistema microencapsulado, tales como contenido de microorganismos, tamaño de partícula y tiempo de germinación han sido estudiados. Las microesferas se prepararon mediante el método de coaservación-separación de fases, utilizando una etapa intermedia de emulsión múltiple. Las condiciones de preparación han sido lo suficientemente benignas para no producir cambios en las propiedades biológicas generales del sistema, pero con la protección que le otorga la matriz del hidrogel, la cual evita la directa comunicación con el medio externo.
La viabilidad demostrada por las microesferas con las formas esporuladas fue significativamente superior a las de las formas vegetativas.
PALABRAS CLAVE: Alginato. *Bacillus subtilis*. Inmovilización. Microesferas.

ABSTRACT

Bacillus subtilis *microspheres were prepared by using sodium alginate. Some typical properties of microencapsulated systems such as content of microorganisms, particle size and germination time were studied. Microspheres are prepared by the coacervation phase separation method, mainly based on an intermediate stage of primary emulsion. The preparation conditions were very soft to avoid changes in general biological products of calcium alginate microspheres containing cells, but giving them protection with a hydrogel matrix, without preventing the communication with the surrounding medium. The encapsulation of microbial spores and viable cells of a model microorganism (*Bacillus subtilis*) can be achieved by using sodium alginate in a satisfactory manner. Spore microspheres showed higher viability comparated to vegetative microspheres.*
KEY WORDS: Alginate. *Bacillus subtilis.* Immobilization. Microspheres.

INTRODUCTION

Microencapsulation has been suggested as an alternative method for entrapment and immobilization of whole cells or their extracts. Currently there is a limited number of reports describing the microencapsulation of microbial cells.

The microencapsulation method in alginate gel is carried out in a single step process under very mild conditions and should be compatible with most living cells.

Alginates are a family of polysaccharides compose of (-L-glucuronic acid (G) and (-D-mannuronic acid (M) residues, arranged in homopolymeric blocks of each type (MM, GG) and in heteropolymeric blocks which are reported to have a major impact on the properties of the different systems.

Some hydrophilic polymers have ion binding properties. Among these are alginates which belong

to a family of unbranched binary copolymers of linked acid residues.

Alginates have the ability to bind multivalent cations being the basis of their gelling properties, leading to the formation of covalent bonds yielding insoluble hydrogels.

This anionic polysaccharide forms strong gels with divalent cations like Ca^{2+}, giving both strength and flexibility. Such crosslinking process stiffens and roughens the polymer and reduces the swelling in solvents.

The soluble sodium alginate was crosslinked with calcium chloride resulting in the formation of the insoluble calcium alginate. Natural polymers are used both as carriers and determinants of the release rate in controlled release systems (Magee 1981).

The main advantages of natural polymers lie in their biocompatibility and biodegradability, without producing systemic toxicity on administration (Pepeljnjak 1988; Takka and Acarturk 1999a).

The objectives of the present study were to investigate the possibility of microencapsulation of a model (non-pathogenic) microorganism (*Bacillus subtilis*) with sodium alginate and to study the viability of the microorganism after the preparation step (Pepeljnjak 1994; Al Musa 1999; Takka and Acarturk 1999b).

MATERIALS

Sodium alginate low viscosity (Sigma Chemical), Ether sulphuric HPLC grade (Merck) and Calcium chloride (Carlo Erba, Milan, Italy).

The non-ionic surfactants used were Sorbitan monooleate (Span 80) and Polyoxyethylene sorbitan monooleate (Tween 80), manufactured by Atlas Co.

Bacterial spores and viable cells of model microorganism (*Bacillus subtilis*, strain 6633) were used as biological materials. Samples were cultivated on neutral nutrient broth at 37 °C. The other chemicals used are of analytical grade. All materials were used as received.

METHODS

Preparation of w/o primary emulsions

The primary emulsions were prepared by one-stage emulsification procedure. The oil external phase, containing 70 mL of liquid vaseline, 30 mL of ether sulfuric, and sorbitan monooleate (3%; w/w) was emulsified in an equal volume of sodium alginate solution (4%; w/v) containing the suspension cells, by means of a small vortex mixer (Whirlimixer) to produce the primary water in oil emulsion.

After preparation, the nature of the emulsions was confirmed by microscopic examination.

Preparation of the microspheres

Microspheres are prepared by the coacervation phase separation method, mainly based on an intermediate stage of primary emulsion.

Alginate gel microparticles are prepared by the crosslinking of sodium alginate, participating in the aqueous internal phase of the primary emulsion with calcium ions ($CaCl_2$ solution 10 % w/w).

The supernatant is decanted, the sediment is washed with distilled water (200 mL) and the system is filtered and dried at 37 ºC, until they reach a constant weight.

The microspheres are prepared as follows: type I (spores) and type II (viable cells) (Lim and Sum 1980).

Morphology

The formation of microspheres was monitored by optical microscopy (Optiphot, Nikon)

Particle size distribution

United States standard sieves ranging from 50 - 700 micra were used to determine the particle size of 10g microspheres (USP 1999). This procedure was repeated three times for different batches (Figures 1 and 2).

FIGURE 1. Particle size distribution of microspheres with viable cells. Data are expressed as the mean for three to five experiments.

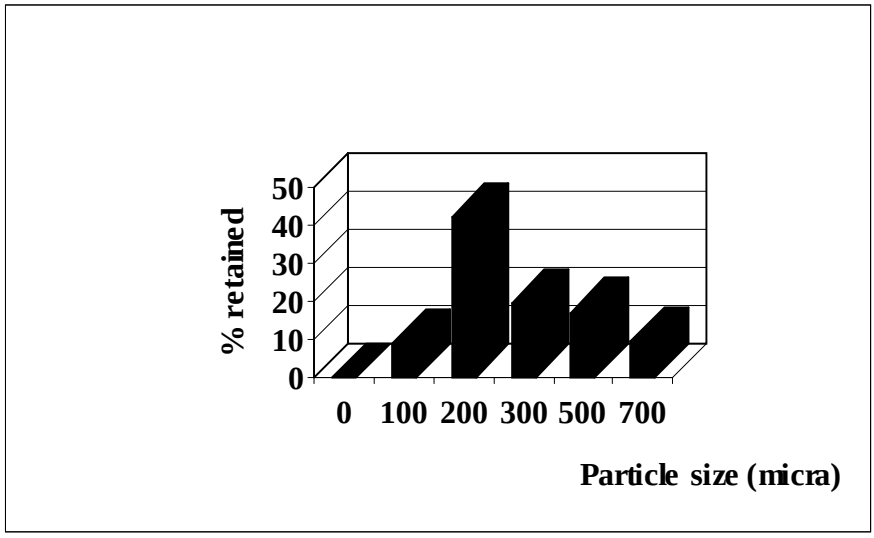
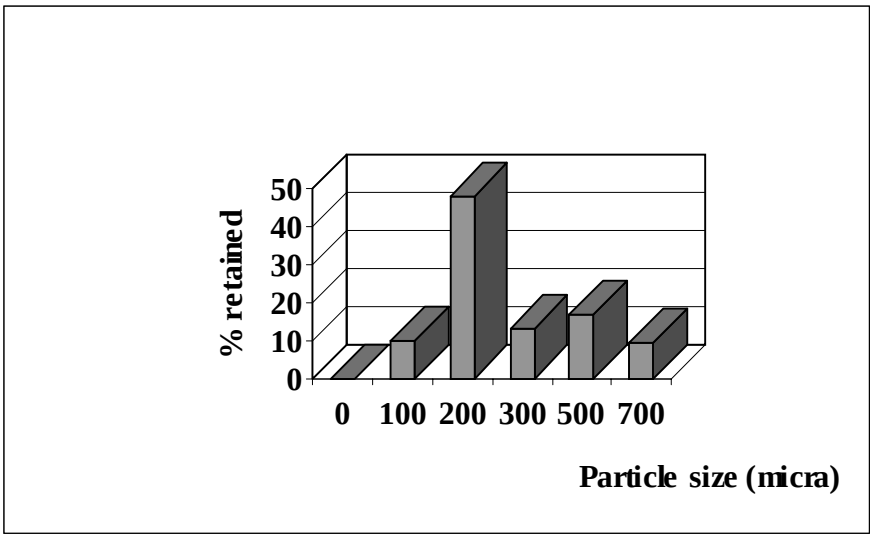


FIGURE 2. Particle size distribution of microspheres with spores. Data are expressed as the mean for three to five experiments.



RESULTS AND DISCUSSION

Two types of microspheres were prepared as follows:

— Type I: spores (alginate concentration 2% w/w).

— Type II: viable cells (alginate concentration 2% w/w).

The microspheres obtained were polydisperse systems with particle size distribution conforming to a normal distribution and with mean diameters between 50 and 300 micra.

The microspheres were well formed and in spite of their soft hydrogel nature they resisted all the manipulations in liquid media during experimental work. However, an increase in microsphere concentration in aqueous media was obtained, but it was not possible to filter the microspherical product.

The difference between microspheres prepared with microbial spores and with viable cells is evident from the data for the average number of cells per microsphere and the germination time (Table I).

TABLE I. Alginate microsphere germination properties including Bacillus subtilis (colony-forming units/g).

Time (days)	Type I Spores (CFU/g)*	Type II Viable cells (CFU/g)*
0	3.1×10^9	2.8×10^9
30	3.0×10^9	8.9×10^7
90	1.2×10^8	4.3×10^7
150	1.0×10^8	3.4×10^7

***** CFU/g **:** colony-forming units/g.

The germination time of encapsulated cells is significantly higher than that of the cells that are not encapsulated (control), giving a relative indication of protection achieved by the process of microencapsulation. After the lag-time due to encapsulation, cell growth was uninhibited and there was no difference between encapsulated and free cells. Viability of encapsulated cells was kept unchanged during of the experiments and since no difference was noticed, it can be presumed that it can be kept for much longer.

Polymerization conditions allow obtaining to obtain microencapsulated cells during 150 days.

CONCLUSIONS

The results of this study showed that the encapsulation of microbial spores and viable cells of a model microorganism can be satisfactorily achieved by using sodium alginate. Very mild conditions during the preparation step allow the production of microspheres containing cells with seemingly no changes in their general biological properties but gives them protection with a soft hydrogel matrix while at the same time it does not prevent completely the communication with the surrounding medium.

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