Obtaining size-controlled microcapsules by ionic gelation with high and low acyl gellans containing *Lactococcus lactis*

Obtención de microcápsulas de tamaño controlado conteniendo *Lactococcus lactis*, utilizando mezclas de gelanos de alto y bajo acilo

Rafael E. González[*]*, Juan A. Salazar*[**]*, Jaime A. Pérez*[*]

Abstract

Control the diameter of microcapsules obtained with functional biopolymer is a crucial parameter in the success of food applications, since it affects the protection of microencapsulated microorganism and also in the texture of the final product. The aim of this study was to assess the obtaining of controlled size microcapsules containing *Lactococcus lactis,* using mixtures of high acyl gellan (HA) and low acyl gellan (LA). A concentration of 0.2% (w/w) gellan was employed using a simple design, generating the following mixtures: 100HA/0.0LA, 0.0HA/100LA, 25HA/75LA, 50HA/50LA and 75HA/25LA. The diameter of the microcapsules, efficiency of microencapsulation and viability of the microencapsulated microorganism were studied in function of the speed of agitation (400-800 rpm) and surfactant concentration (sorbitan monooleate) (0.0-0.2%)v/v. The results indicated that mixtures with concentration equal or greater than 50% of HA gellan are not efficient for obtaining microcapsules, only the LA gellan and the mixture 25HA/75LA gave acceptable results. The viability of the microorganism and the efficiency of microencapsulation were descending function of the stirring speed and surfactant concentration. The microcapsules obtained had diameters not greater than 80 µm when the highest concentrations of surfactant (0.2% v/v) and stirring speed (800 rpm) were used, suggesting that the ionic gelation can be used to obtain microcapsules of controlled size (15-75 µm) containing *Lactococcus lactis* with high viability (83.32%) and high efficiency of microencapsulation (82.4%), which makes it feasible for use in food applications.

Key words: microencapsulation, high acyl gellan, low acyl gellan, ionic gelation, *Lactococcus lactis*.

Resumen

Controlar el diámetro de microcápsulas obtenidas con biopolímeros funcionales es un parámetro crucial en el éxito de aplicaciones alimentarias, ya que influye en la protección del microorganismo microencapsulado y también en la textura del producto final. El objetivo de este trabajo fue evaluar la obtención de microcápsulas de tamaño controlado conteniendo *Lactococcus lactis,* utilizando mezclas de gelana de alto (HA) y bajo acilo (LA). Se empleó una concentración de gelana de 0.2% p/p usando un diseño de mezclas simple, generando las siguientes mezclas, 100HA/0.0LA, 0.0HA/100LA, 25HA/75LA, 50HA/50LA, 75HA/25LA. El diámetro de las microcápsulas, la eficiencia de microencapsulación y la viabilidad del microorganismo microencapsulado fueron estudiadas en función de la velocidad de agitación (400-800 rpm) y concentración de surfactante (sorbitan monooleate) (0.0-0.2%)v/v. Los resultaron indicaron que las mezclas con concentración igual o superior al 50% de gelana de HA, no son eficientes para obtener microcápsulas; solamente dieron resultados aceptables la gelana de LA y la mezcla 25HA/75LA. La viabilidad del microorganismo y la eficiencia de microencapsulación variaron en función descendente de la velocidad de agitación y concentración de surfactante. Las microcápsulas obtenidas no presentaron diámetros superiores a 80 µm cuando se emplearon las mayores concentraciones de surfactante (0.2%) y velocidad de agitación (800 rpm), sugiriendo que la gelación iónica puede ser utilizada para obtener microcápsulas de tamaño controlado (15-75 µm) conteniendo *Lactococcus lactis* con alta viabilidad (83.32%) y eficiencia de microencapsu-

* Biotechnology PhD, Faculty of Engineering, University of Cartagena, Piedra de Bolívar – Av. Del Consulado, Calle 30 N° 48-157, Cartagena de Indias, D.T. y C – Colombia. rgonzalezc1@unicartagena.edu.co

** Engineering PhD, Department of Biotechnology and Bioengineering, CINVESTAV-IPN. Av. IPN 2508. P.O. Box 14-740, CP 07360, D.F., México.

lación (82.4%), cuando se utiliza la mezcla 25HA/75LA a 800 rpm y 0.2% v/v de surfactante, lo cual la hace factible para su uso en aplicaciones alimentarias.

Palabras clave: microencapsulación, gelana de alto acilo, gelana de bajo acilo, gelación iónica, *Lactococcus lactis*.

Recibido: diciembre 10 de 2012 **Aprobado:** noviembre 12 de 2013

Introduction

Microencapsulation is a process by which certain bioactive substances are retained within a matrix or wall system in order to protect them from deleterious environmental conditions, prevent loss and gradually release them under controlled conditions (Anal and Stevens, 2005; Anal *et al*., 2006; Kailasapathy and Masondole, 2005; Yáñez *et al*., 2002). A microcapsule consists of a strong, thin, semi-permeable and spherical membrane around a solid or liquid core with a diameter that varies from a few microns to 1 mm (Goncalves *et al*., 1992).

There are many techniques to carry out the microencapsulation of various compounds and microorganisms, among which there may be mentioned spray drying, extrusion, fluidized bed, simple or complex coacervation, liposomes, inclusion in complexes (Gouin, 2004), spray coating, interfacial polymerization, and ionic gelation (Thies, 1996), the latter being a developed process for immobilizing a cell, which uses an anionic polymer mainly alginate as component of the membrane, in combination with divalent ions such as calcium to induce gelation (King, 1988). Some of the drawbacks that have in the production of microcapsules are the so large diameters of microcapsules that are obtained (Adikhari *et al*., 2003; Poncelet *et al*., 1992; Moslemy *et al*., 2004) obtained by internal gelation microcapsules of gellan with diameters between 34 and 265 µm as a decreasing function of the stirring speed, concentration of surfactant and emulsion time.

The average diameter of the microcapsules can be an important factor in the stability and efficiency in microencapsulation, i.e. large microcapsules generally provide greater protection than the smaller diameter microcapsules, but have a poor dispersion in the final food product. However, small diameter microcapsules have low microencapsulation efficiency. Therefore, there must be an optimum diameter of microcapsules, not only for a good protection of the microorganism but for a good distribution of the microcapsules in the food matrix used (Zhao *et al*., 2008)

In the food area, lactic acid bacteria (LAB) are widely used in fermented products (Fanema, 1996). Among the LAB, *Lactococcus lactis* is the main microorganism used by many industries as starter culture in manufacturing a wide range of dairy products such as cheese, fermented milks and creams, which are important due to the technological, nutritional and beneficial properties to the health of consumers that they have (Stanton *et al*., 2001).Thus the microencapsulation of this bacterium is of great importance for food applications, since it is a technique that involves the use of non-toxic food grade components such as K-carrageenan, alginate, mesquite gum and gellans because these gums produce soft microcapsules suitable for use in food systems. (Audet *et al*., 1991; Kailasapathy, 2002; Özer *et al*., 2009).

However, the use of gellan gum as encapsulating matrix for the protection of microorganisms has been recommended in fermentation processes because of its mechanical and thermal stability. Norton and Lacroix (1990) and Camelin *et al*. (1993) found that gellan gels can provide mechanical stability to the matrix for the immobilization of *Bifidobacterium longum* (the gels were stable after 150 h of fermentation in serum), also the biocatizalyzed activity (production of lactic acid) was much greater compared with the values reported for *B. longum* entrapped in K-carrageenan. The gellan gum is an anionic linear polysaccharide gel-forming secreted by the bacterium *Sphingomonas paucimobilis* (formerly *Pseudomonas elodea*) (Banik *et al*., 2000) approved for use in foods by the FDA in 1992 (Pszczola, 1993). It consists of monosaccharaides β-1,3-D-glucose, β-1,4-D glucuronic acid and α-1,4-L-rhamnose in molar ratios 2:1:1 (Chandrasekaran and Radha, 1995; Jansson *et al*., 1983). In the native gellan also called high acyl gellan (HA) the glucose residue A contains a substitution of a L-glycerate group at C_2 and an acetate group to a substitution level of approximately 50% in the C_6 (Kuo and Mort,1986).

By contrast, the low acyl gellan (LA) is produced by removal of acetate and glycerate groups with a strong alkali treatment at high temperatures. This structural difference between the two gellan molecules causes a great disparity in the rheological and functional properties of the two biopolymers (Mao *et al*., 2000). So they

are also used as thickeners or gelling agents in various food applications and biotechnology. Both gellan molecules form a three dimensional net under appropriate aqueous conditions (Chandrasekaran *et al*., 1988).

Gellan gum is used for the microencapsulation of viable cells at lower concentrations than those are normally employed when using alginate, K-carrageenan and agar (Buitelaar *et al*., 1988; Nilsson *et al*., 1983). It has been reported that gellan is capable of forming gels at low concentrations (0.05%); however, concentrations commonly used in the food industry are in a range between 0.2 to 0.4% (Kelco International, 1991). Therefore, the gellan gum can be an attractive alternative in the microencapsulation of bacteria of interest in food using low concentrations of the biopolymer. It is important to mention that gellans are resistant to high temperature, extreme acidic conditions (Brownsey *et al*., 1984; Jay *et al*., 1998) and has better technical properties for the microencapsulation than other biopolymers as alginate (Sun and Griffiths, 2000).

The main objective of this study was to evaluate the possibility of obtaining controlled size microcapsules containing *Lactococcus lactis* using binary mixtures of gellan evaluating microencapsulation parameters such as efficiency, viability of lactic acid bacteria and size of the microcapsules obtained, may be representing an useful alternative in food applications.

Materials and Methods

Microencapsulation

Preparation of dispersions

The dispersions were prepared separately in deionized water at a concentration of 0.2% w/w, using a simple design of mixtures of HA, LA gellan and the mixtures 25HA/75LA; 50HA/50LA; 75HA/25LA. Subsequently calcium was added (30 mM) (Huang *et al*., 2003) and they are dispersed by constant stirring at 90°C/ 10 min.

Preparation of emulsion

For the experimental design an orthogonal design L9 3^2 was used where the factors were the concentration of surfactant (0.0 – 0.2% v/v) and the stirring speed (400 – 800 rpm), because these parameters are those that most affect the size of the microcapsules (Krasaekoopt *et al*., 2003; Moslemy *et al*., 2004). From the dispersions of biopolymers containing the inoculum in a concentration of 10^8cells/ml of *Lactococcus lactis*, emulsions were prepared with the addition of Span® 80 to the vegetable oil under constant agitation. Gluconolactone is then added until a pH of 4.2 to initiate the gelation process of the gellans. Finally the oil is removed by adsorption and the microcapsules contained in the aqueous phase are centrifuged at 5000 rpm / 10 min twice with saline solution and stored at 4 °C until its use.

Physical examination of the microcapsules

Electron Microscopy

The microcapsules are immersed in a solution of 2.5%v/v glutaraldehyde in phosphate buffered saline (abbreviated PBS) (pH 7.3) for 1 hour at room temperature. Subsequently 3 rinses in PBS for 5 minutes each are performed, the samples are dehydrated with ethanol 50, 60, 70, 80, 90 and two changes of 100% for 15 minutes at each concentration. Then, the samples were dried at the critical point in the presence of carbon dioxide using a Samdri-780 dehydrator (Tousimis, USA). Subsequently, a gold coating was deposited by a DESK II gold sputter machine (Denton Vacuum, USA) over 1 min. Finally, the microcapsules were observed using a SEM-Scanning Electron Microscope, JSM-35C® (JEOL, Germany); the microcapsules are fixed with double sided adhesive tape commonly used in SEM observations.

Efficiency of microencapsulation

The suspension of microcapsules is centrifuged to separate the free cells, then the bacterial concentration in the supernatant is determined and the encapsulation efficiency is calculated as well:

$$EE\ (\%) = (A-B)/A \times 100$$

Where A is the total bacteria concentration in the suspension, and B is the concentration of unencapsulated bacteria found in the supernatant (Capela *et al*., 2007).

Determination of viability of *Lactococcus lactis*

Lactococcus lactis viability is determined in a flow cytometer. Suspensions of known concentrations of live and dead microorganisms were analyzed to standardize the technique as described by Molecular Probes (2001). Subsequently, is determined the viability of microencapsulated microorganisms. This technique is based on the intersection of the microorganisms and / or microcapsules with an argon laser at 488 nm, that

generates fluorescence which enable to distinguish between live microorganisms and damaged microorganisms due to the reactivity of the fluorochromes. The samples were previously incubated in the dark for a time of 20 minutes in presence of fluorochromes (SYTO®9 and propidium iodide) in a ratio (1:1). Bacterial suspensions and stained microcapsules suspensions were placed in the cytometer chamber and when the laser passed through the suspension, fluorescent signals were emitted that were separated according to their wavelengths. These signals are integrated and analyzed with the computer program Summit ® WM Software Version 5.1 (Extreme Networks, Inc. California) generating percentages of live and damaged microorganisms according to the fluorescence emission from the fluorochromes, subsequently the emission response area is divided into three zones, where R1 corresponds to dead microorganisms, R2 corresponds to live microorganisms and finally R3 that represents inactive material samples to the fluorochromes.

Statistical analysis

Differences between mean values of the viability and efficiency of microencapsulation were determined with the Tukey's test ($p<0.05$) and one way analysis of variance (ANOVA) was performed using the software SPSS ver. 13.0. All determinations were done in triplicate.

Results and Discussion

Formation of Microcapsules

The microencapsulation method used consists in forming an emulsion between two phases, one hydrophobic and one hydrophilic, where by agitation, a great number of drops are originated which are gelled by acidification with gluconolactone, since calcium is released by substitution reaction of the salt used ($CaCO_3$). In this study microcapsules were obtained with the mixture 25HA/75LA and LA gellan with a 1:2 water/oil rate, under varying conditions of stirring speeds (400, 600 and 800 rpm) and surfactant concentrations (0.0, 0.1 and 0.2%v/v), where it was allowed the separation of the phases by applying a centrifugal force (5000 rpm/10min) at the required time.

Binary mixtures of 25HA/75LA gellan with concentrations higher than 50% of HA gellan were not efficient in obtaining microcapsules, due to the formation of a gel at high temperatures (70º C) (Matsukawa and Watanabe, 2007) which prevented the formation of the emulsion, making it easier obtaining elastic gels (Sanderson *et al*., 1988), with which it is not possible to maintain the shape of the microcapsules; precluding the microencapsulation process. Microcapsules were obtained only with the LA gellan and the 25HA/75LA mixture because LA gellan provides gels firmer than the HA gellan (Sanderson, 1990). One possible explanation for this phenomenon is that the HA gellan needs higher temperature for the conformational order than LA gellan (Morris *et al*., 1996), this is probably due to the presence of the acyl groups present in the HA gellan, preventing the association between the biopolymer chains (Chandrasekaran and Thailambal, 1990).

Microencapsulation

The microcapsules obtained in all experiments performed in this study showed a unimodal behavior, this behavior may be explained by the slow release of calcium ions from calcium carbonate because of disruption of the gluconolactone (Pelaez and Karel, 1981), to place into the carboxyl groups of gellan, initiating the gelation process (Larwood *et al*., 1996; Tang *et al*., 1997), having sufficient time to have the size and shape of the final microcapsules.

Some parameters which are evaluated in the selection of materials used in the microencapsulation are efficiency, stability under storage conditions and the observing surface by scanning electron microscopy, among other observations (Pérez *et al*., 2003). Hence the microcapsules were observed in the scanning electron microscope, showing the existence of uneven and irregular structures and surfaces jagged in the microcapsules obtained with LA gellan (figure 1b),while the microcapsules obtained with the mixture of 25HA/75LA gellan (figure 1a) showed a less spherical structure with more irregular surfaces than those mentioned above; however, these results are within the afore mentioned types of microcapsules by Shahidi and Han (1993) and by Gibbs *et al*. (1999). Note that the microcapsules prepared with surfactant had a higher fragility (Graphs not shown) during the dehydration process with alcohol and critical point drying, which indirectly indicates a high solubility in polar substances such as alcohol and thus a weak capsular membrane (Yáñez, 2007).

Regarding the diameters of microcapsules, figure 2 shows the distributions of the microcapsules obtained with mixtures of HA/LA gellan and LA gellan under changing conditions of surfactant and stirring speed, where it is observed that the largest diameter distribution is presented in the microcapsules obtained with LA gellan without surfactant at 400 rpm (Figure 2a); where, the distribution of diameters ranges values

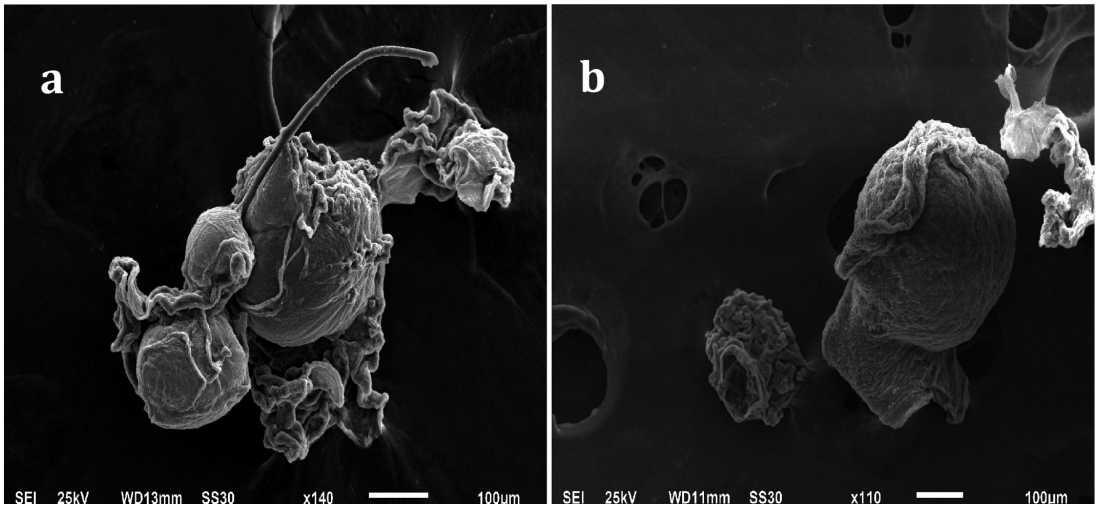

Figure 1. Micrographs obtained by scanning electron microscopy of microcapsules obtained by mixing gelanas 25HA/75LA (a) and low acyl gellan (b)

from 15 to 250 µm, while the microcapsules obtained with LA gellan at higher stirring speeds (800 rpm) and surfactant concentrations (0.2%) showed values from 15 to 75 µm (figure 2c),observing a diminution in diameter of thereof with increasing concentration of the surfactant and the stirring speed; the average diameter of the microcapsules obtained under these conditions was 13.72 µm, being a diameter similar to that reported by Homayouni *et al*. (2008) who reported that microcapsules with a diameter of 17.89 µm increase survival in a 30% of *Lactobacillus casei* and *Bifidobacterium lactis* in ice cream.

Likewise the microcapsules obtained with mixtures 25HA/75LA showed the same behavior, i.e. with increasing surfactant concentration and stirring speed (Figures 2d, 2e and 2f) decreased the diameter thereof. The highest frequencies for the microcapsules obtained with mixture of 25HA/75LA were obtained for the diameters of between 15 and 80 µm (Figure 2f), which represents the ideal size for food applications, since a smaller diameter results in decreased cell protection and a larger diameter may result textural defects in the food product in which they are used (Lacroix *et al*., 2005).

By contrast when higher stirring speeds and surfactant concentrations were used, the highest frequencies were between 15 and 75 µm and this similar behavior reported by Kim, *et al*. (2008) who obtained diameters of 75 µm in microcapsules prepared with sodium alginate, note that microcapsules of diameters less than 80 µm are desired at the industrial level because they do not affect the sensory properties of foodstuffs in which they are employed (Tyle, 1993).

The microcapsules obtained in this work at lower stirring speeds have diameters between 15 and 250 µm, which are also within the optimum diameters reported by Robitaille *et al*. (1999) whereby the standard diameter for microcapsules should be <350 µm considering only the size of the dairy organism (0.6-0.9 µm x 1.5 µm). Similarly, in this work are reported lower diameters than those of Jankowski *et al*. (1997), who obtained microcapsules with diameters between 5.2 and 5.7 mm by means of the coacervation technique using sodium alginate and potato starch as covering material; also the diameters are lower than those published by Groboillot *et al*. (1993) who used chitosan to encapsulate *Lactococcus lactis* achieving an increase in the diameter of the microcapsules from 220 to 629 µm by increasing the concentration of chitosan from 1 to 4%; this difference in sizes can be explained since the concentration of gellan over microencapsulation process (0.2%) was kept constant in this work.

In general, it can be seen that the microcapsules obtained for both the mixture with 25HA/25LA and LA gellan by increasing the concentration of surfactant, the size of the microcapsules decreases (Poncelet *et al*., 1990) this is due to diminution in the surface tension leading to a better emulsion (Adamson, 1982), coupled with increased stirring speed, facilitates disruption of the droplets (Moslemy *et al*., 2004) causing small microcapsules. In this work the surfactant concentration was limited to 0.2% because of its potential toxic effect.

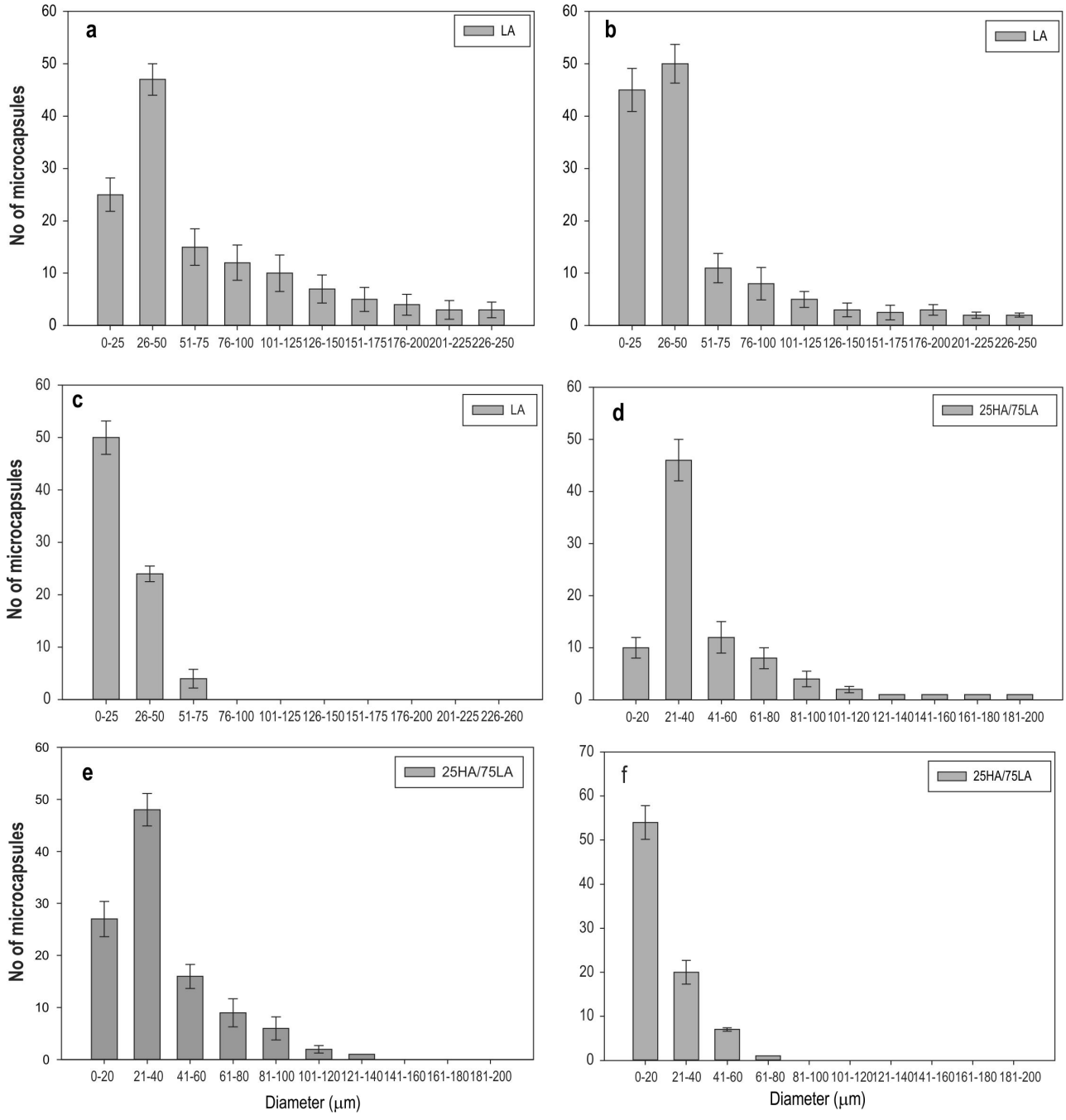

Figure 2. Average diameter distribution of the microcapsules obtained with low acyl gellan (LA) and mixtures thereof (25HA/75LA) at different concentrations of surfactant and stirring speeds. (a, d: 400 rpm, b, e: 600 rpm y 0.1% surfactant; c, f: 800 rpm y 0.2% surfactant).

With regard to the microencapsulation efficiency, the microcapsules obtained with the mixture 25HA/75LA showed the highest average percentages (92.5%) compared with those obtained with LA gellan (86.9%), this can be because the mechanism of gelling of the HA gellan does not require calcium ions to gel, it needs only to be subjected to heating in order to initiate a cross-linking between gellans chains, which is started with the temperature drop (Banik *et al.*, 2000). It was noted that efficiency is a descendant function of the stirring speed and surfactant concentration, since there were differences statistically significant (p<0.05) for all

treatments applied (table 1), these results are contrary to those published by Gharsallaoui *et al*., (2007) where it is mentioned that the lack of interfacial properties leads to an increase in the percentage of microencapsulation. This decrease in efficiency can be attributed to some organisms may become trapped in the oil when preparing the microcapsules.

Viability of *Lactococcus lactis*

A method for distinguishing and quantifying live and dead microorganisms was employed with the aid of a flow cytometer, even in mixtures containing both types of microorganisms. This method uses a mixture of two fluorochromes that stain the nucleic acids and which are excited when exposed to an argon laser at 488 nm which is the cytometer (Molecular Probes, 2001), this permits the analysis of the bacteria on the surface and inside the microcapsules (Demirci *et al*., 2003; Yáñez *et al*., 2008). Figure 3 shows typical graphs obtained by the red fluorescence versus green fluorescence.

After microencapsulation the number of live microorganisms of *Lactococcus lactis* decreased approximately 11%, taking into account all the treatments applied at the time of microencapsulation. This result probably took place because of stirring speeds used; suggesting the effect of shear rate on the survival of the microorganism (Arnaud *et al*., 1992; Capela *et al*., 2007). However, the number of microencapsulated microorganisms can increase compared to free microorganisms when used in food fermentations (Kailasapathy, 2006; Krasaekoopt *et al*., 2006).

The samples which contained only *Lactococcus lactis* showed 97.32% of viability (figure 3a). With respect to the results of microencapsulation and after applying the ANOVA (one way) there were differences statistically significant (p<0.05) among all the viabilities obtained, highlighting the effect that both the stirring speed as the presence of surfactant have on this parameter.

The microcapsules obtained with the mixture 25HA/75LA 400 rpm without surfactant have the highest viability (91.23%) (figure 3d) while the low acyl microcapsules obtained at 800 rpm and 0.2% surfactant (figure 3c) showed the lowest viability (83.32%). Viability percentages obtained showed a decrease in bacterial viability of 10.32% (figures 3b and 3c) in the microcapsules of LA gellan and 7.91% (figures 3d and 3e) for microcapsules obtained with the mixture HA/LA, suggesting an inverse relationship between increasing the stirring speed and concentration of the surfactant with microbial viability.

The cellular microencapsulation has a protective effect on bacteria when they are introduced in food systems, so it is possible to apply the microcapsules obtained in this work to food systems because high percentages of microencapsulation were obtained (> 80%), which are larger to those reported by Yáñez *et al*. (2008) who obtained viabilities of 46.7% in the microencapsulation of *Lactobacillus spp*. using a mixture of gellan and

Table 1. Efficiency of gellans microcapsules obtained at different concentrations of surfactant and stirring speeds.

GELLANS	% SPAN	RPM	%EE	GELLANS	% SPAN	RPM	%EE
HA/LA	0.0	400	98.2±0.14^{a}	LA	0.0	400	97.8±0.26^{a}
		600	97.3±0.18^{b}			600	96.5±0.32^{b}
		800	92.6±0.24^{c}			800	89.2±0.45^{c}
HA/LA	0.1	400	96.3±0.21^{d}	LA	0.1	400	91.8±0.41^{d}
		600	93.1±0.38^{e}			600	85.2±0.18^{e}
		800	87.2±0.15^{f}			800	78.3±0.32^{f}
HA/LA	0.2	400	94.8±0.31^{g}	LA	0.2	400	87.3±0.12^{g}
		600	90.6±0.26^{h}			600	84.2±0.11^{h}
		800	82.4±0.32^{i}			800	72.2±0.28^{i}

Columns with no common letter differ significantly at a confidence level of 95%

mesquite gum; this marked difference in the viabilities is due to a technique using a cross-linking agent such as glutaraldehyde, which may affect the viability of the microorganism. However, ionic gelation is a technique originally designed for the cells microencapsulation, thus results with high viability are obtained in comparison with those obtained by other authors (Champagne *et al*., 1992).

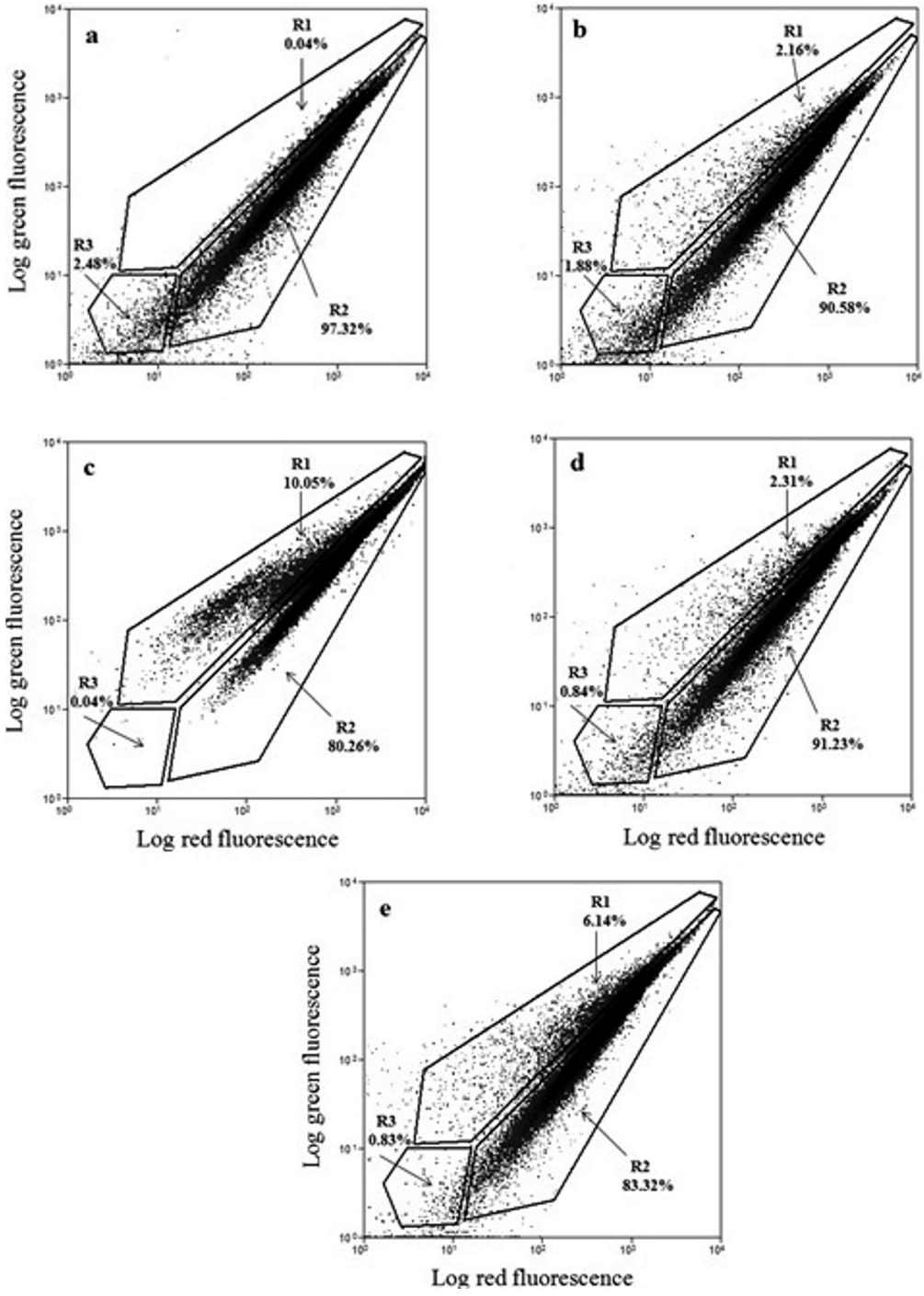

Figure 3. Viability obtained by flow cytometry in *Lactococcus lactis* microencapsulated using gellan (a: *Lactococcus lactis*, b: *Lactococcus lactis* microencapsulated with LA gellan to 400 rpm, c: *Lactococcus lactis* microencapsulated with LA gellan to 800 rpm and 0.2% of surfactant; d: *Lactococcus lactis* microencapsulated using gellans mixtures 25HA/75LA to 400 rpm; e: *Lactococcus lactis* microencapsulated using gellans mixtures 25HA/75LA to 800 rpm and 0.2% surfactant).

Conclusions

Under the conditions studied it was not possible to obtain microcapsules with HA gellan and/or mixtures with concentrations equal to or greater than 50%. The microcapsules of 25HA/75LA mixtures showed higher average microencapsulation efficiency (92.5%) and percentage of viability (86.84%) than those obtained with low acyl gellan (86.9 and 84.98%, respectively). With regard to the parameters evaluated, both the stirring speed as the concentration of surfactant showed to be descending functions on the size of the microcapsules, microencapsulation efficiency and microbial viability.

It is possible to obtain microcapsules with mixtures of 25HA/75LA gellan and LA gellan with diameters between 15 and 80 µm using stirring speeds of 800 rpm and surfactant concentrations of 0.2%v/v, with a size suitable for food applications; therefore, results of this study suggest that the ionic gelation can be used to provide microcapsules of controlled size containing *Lactococcus lactis* with viabilities and microencapsulation efficiencies greater than 70% which makes it ideal for food applications. Experiments using dairy product are currently underway in our laboratory.

Acknowledgements

The authors wish to thank CONACyT for the scholarship No. 296286 to REGC, and Miguel Márquez for technical support and Walmy Cuello Jimenez for all the help during the english editing

Bibliographic references

Adamson A. 1982. *Physical chemistry of surfaces*. New York: Wiley Inc. pp.24-87.

Adikhari K., Mustapha A., Grun I. 2003. Survival and metabolic activity of microencapsulated Bifidobacterium in stirred Yoghurt. *Journal Food Science*. 68 (1): 275-280. DOI: 10.1111/j.1365-2621.2003.tb14152.x

Anal A.K., Stevens W.F. 2005. Chitosan-alginate multilayer beads of controlled release of ampicillin. *International Journal of Pharmaceutics*. 290(1): 45-54. DOI:10.1016/j.ijpharm.2004.11.015

Anal K., Stevens W.F., Remuñán-López C. 2006. Ionotropic cross-linked chitosan microspheres for controlled release of ampicillin. *International Journal of Pharmaceutics*. 312 (1): 166-173. DOI:10.1016/j.ijpharm.2006.01.043

Arnaud J.P., Lacroix C., Choplin L. 1992. Effect of agitation rate on the cell release rate and metabolism during continuous fermentation with entrapped growing. *Biotechnology Techniques*. 6(3): 265-270. DOI: 10.1007/BF02439356

Audet P., Paquin C., Lacroix C. 1991. Effect of medium and temperature of storage on viability of lactic acid bacteria immobilized in K-carrageenan locust bean gum gel beads. *Biotechnology Techniques*. 5(4): 307-312. DOI: 10.1007/BF02438669

Banik R.M., Kanary B., Upadhyay S.N. 2000. Exopolysaccharide of the gellan family: prospects and potential review. *World Journal of Microbiology and Biotechnology*. 16(5): 407-414. DOI: 10.1023/A:1008951706621

Brownsey G.J., Chilvers G.R., Anson K.I., Morris V.J. 1984. Some observations (or problems) on the characterization of gellan gum solutions. *International Journal of Biological Macromolecules*. 6(4): 211-214. DOI:10.1016/0141-8130(84)90033-3

Buitelaar R.M., Hulst A.C., Tramper J. 1988. Immobilization of biocatalysts in thermogels using the resonance nozzle for rapid drop formation and an organic solvent for gelling. *Biotechnology Techniques*. 2(2): 109-114. DOI: 10.1007/BF01876160

Camelin I., Lacroix C., Paquin C., Prévost H., Cachon R., Divies C. 1993. Effect of chelants on gellan gum rheological properties and setting temperature for immobilization of living Bifidobacteria. *Biotechnology Progress*. 9(3): 291-297.

Capela P., Hay T.K.C., Shah N.P. 2007. Effect of homogenization on bead size and survival of encapsulated probiotic bacteria. *Food Research International*. 40(10):1261-1269. DOI:10.1016/j.foodres.2007.08.006

Champagne C.P., Gaudy C., Poncelet D., Neufeld R.J. 1992. *Lactococcus lactis* release from calcium alginate beads. *Applied Environmental Microbiology*. 58(5): 1429-1434

Chandrasekaran R., Puigjaner L., Joyce K.L., Arnott S. 1988. Cation interactions in gellan: An X-ray study of the potassium salt. *Carbohydrate Research*. 181: 23-40.

Chandrasekaran R., Thailambal V.G. 1990. The influence of calcium ions, acetate and L-glycerate groups on the gellan double-helix. *Carbohydrate Polymers*. 12(4): 431-442. DOI:10.1016/0144-8617(90)90092-7

Chandrasekaran, R., Radha, A. 1995. Molecular architectures and functional properties of gellan gum and related polysaccharides. *Trends in Food Science and Technology*. 6(5): 143-147. DOI:10.1016/S0924-2244(00)89022-6

Demirci A., Cotton J.C., Pometto A.L, Harkins K.R., Hinz P.N. 2003. Resistance of *Lactobacillus casei* in plastic-composite-support Biofilm reactors during liquid membrane extraction and optimization of the lactic acid extraction system. *Biotechnology and Bioengineering*. 83(7): 749-759.

Fanema O. 1996. *Food Chemistry*, 3rd edition. New York: Marcel Deckker Inc. pp. 23-45.

Gharsallaoui A., Roudaut G., Chambin O., Voilley A., Saurel R. 2007. Applications of spray-drying in microencapsulation of food ingredient: An overview. *Food Research International*. 40(9): 1107-1121. DOI:10.1016/j.foodres.2007.07.004

Gibbs B., Kermasha, S., Alli I., Mulligan, C. 1999. Encapsulation in the food industry: A review. *International Journal of Food Sciences and Nutrition*, 50(3): 213-224.

Goncalves L.M., Barreto M.T., Xavier A.M., Carrondo M.J., Klein J. 1992. Inert support for lactic acid fermentation - a technological assessment. *Applied Microbiology and Biotechnology*. 38(3): 305-311. DOI: 10.1007/BF00170077

Gouin S. 2004. Microencapsulation: industrial appraisal of existing technologies and trends. *Trends in Food Science and Technology*. 15(7-8): 330-347. DOI:10.1016/j.tifs.2003.10.005

Groboillot A, Champagne C, Darling G, Poncenlet D., Neufeld R. 1993 Membrane formation by interfacial cross-linking of chitosan for encapsulation of *Lactococcus lactis*. *Biotechnology and Bioengineering*. 42(10):1157-1163. DOI: 10.1002/bit.260421005

Homayouni A., Azizi A., Ehsani M., Yarmand M.S., Razavi S. 2008. Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. *Food Chemistry*. 111(1): 50-55. DOI:10.1016/j.foodchem.2008.03.036

Huang Y., Tang J., Swanson B., Rasco B. 2003. Effect of calcium concentration on textural properties of high and low acyl mixed gellan gels. *Carbohydrate polymers*. 54(4): 517-522. DOI: 10.1016/S0144-8617(99)00108-3

Jankowski T., Zielinska M., Wysakowska A. 1997. Encapsulation of lactic acid bacteria with alginate/starch capsules. *Biotechnology Techniques*. 11(1):31-34. DOI: 10.1007/BF02764447

Jansson P., Lindberg B., Sandford P. 1983. Structural studies of gellan gum, an extracellular polysaccharide elaborated by *Pseudomonas elodea*. *Carbohydrate Research*. 124(1): 135-139. DOI:10.1016/0008-6215(83)88361-X

Jay J., Colquhoun J., Ridout M., Brownsey J., Morris V., Fialho A., Leitão J., Sá-Correia I. 1998. Analysis of structure and function of gellans with different substitution patterns. *Carbohydrate Polymers*. 35(3): 179-188. DOI: 10.1016/S0144-8617(97)00241-5

Kailasapathy K. 2002. Microencapsulation of probiotic bacteria: technology and potential applications. *Current issues in intestinal microbiology*. 3(2): 39-48.

Kailasapathy K., Masondole L. 2005. Survival of free and microencapsulated *Lactobacillus acidophilus* and *Bifidobacterium lactis* and their effect on texture of feta cheese. *Australian Journal of Dairy Technology*. 60(3): 252-258.

Kailasapathy K. 2006. Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. *LWT-Food Science and Technology*. 39(10): 1221-1227. DOI:10.1016/j.lwt.2005.07.013

Kelco International 1991. Kelco international kelcogelgellan gum. London: Kelco Division of Merck and Co. Inc., Kelco International

Kim, S., Cho, S., Kim, S., Song, O., Shin, I., Cha, D., Park, H. 2008. Effect of microencapsulation on viability and other characteristics in *Lactobacillus acidophilus* ATCC 43121. *LWT-Food Science and Technology*. 41(3): 493-500. DOI:10.1016/j.lwt.2007.03.025

King A. H. 1988. Flavor encapsulation with alginates. *Flavor Encapsulation*. Washington (DC): Risch S.J., Reineccius G.A. Eds. American Chemical Society. Pp. 122-125.

Krasaekoopt W., Bhandari B., Deeth H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. *International Dairy Journal*. 13(1): 3-13. DOI: 10.1016/S0958-6946(02)00155-3

Krasaekoopt W., Bhandari B., Deeth H. 2006. Survival of probiotics encapsulated in chitosan-coated alginate beads in yoghurt from UHT- and conventionally treated milk during storage. *LWT-Food Science and Technology*. 39(2): 177-183. DOI:10.1016/j.lwt.2004.12.006

Kuo M.S., Mort A. 1986. Identification and location of L-glycerate, an unusual acyl substituent in gellan gum. *Carbohydrate Research*. 156(15): 173-187.

Lacroix C., Grattepanche F., Doleyres Y., Bergmaier D. 2005. Immobilised cell technologies for the dairy industry. In *Applications of cell immobilisation biotechnology*. Netherlands: Springer. pp 295-319. DOI: 10.1007/1-4020-3363-X_18

Larwood V., Howlin B., Webb G. 1996. Solvation effects on the conformational behavior of gellan and Calcium ion binding to gellan double helices. *Journal of Molecular Modeling*. 2(6): 175-182. DOI: 10.1007/s0089460020175

Mao R., Tang J., Swanson B. 2000. Texture properties of high and low acyl mixed gellan gels. *Carbohydrate polymer*. 41(4): 331-338. DOI: 10.1016/S0144-8617(99)00108-3

Matsukawa S., Watanabe T. 2007. Gelation mechanism and network structure of mixed solution of low-and high-acyl gellan studied by dynamic viscoelasticity, CD and NMR measurement. *Food Hydrocolloids*. 21(8): 1355-1361. DOI:10.1016/j.foodhyd.2006.10.013

Morris E.R., Gothar M.G., Hember M.W., Manning C., Robinson G. 1996. Conformational and rheological transitions of welan, rhamsan and acylated gellan. *Carbohydrate Polymers*. 30(2): 165-175. DOI:10.3168/jds.S0022-0302(85)81041-9

Molecular Probes. 2001. Live/dead Bac*L*ight Bacterial Viability and Counting Kit (L34856) Product information

Moslemy P., Guiot S., Neufeld R. 2004. Activated sludge encapsulation in gellan gum microbeads for gasoline biodegradation. *Bioprocess and Biosystems Engineering*. 26(4):197-204. DOI: 10.1007/s00449-004-0360-6

Nilsson K., Birnmaum S., Flygare S., Linse L., Schröder U., Jeppsson U., Larsson P., Mosbach K., Brodelius P. 1983. A general method for the immobilization of cells with preserved viability. *European Journal of Applied Microbiology Biotechnology*. 17(6): 319-326. DOI: 10.1007/BF00499497

Norton S., Lacroix C. 1990. Gellan gum gel as entrapment matrix for high temperature fermentation processes-rheological study. *Biotechnology Techniques*. 4(5): 351-356. DOI: 10.1007/BF00157435

Özer B., Kirmaci H., Senel E., Atamer M., Hayaloglu A. 2009. Improving the viability of *Bifidobacterium bifidum* BB-12 and *Lactobacillus acidophilus* LA-5 in white-brined cheese by microencapsulation. *International Dairy Journal*. 19(1): 22-29. doi:10.1016/j.idairyj.2008.07.001

Pelaez C., Karel M. 1981. Improved method for preparation of fruit-simulating alginate gels. *Journal of Food Processing and Preservation*, 5(2): 63-81. DOI: 10.1111/j.1745-4549.1981.tb00622.x

Pérez C., Báez J., Beristain C., Vernon C., Vizcarra M. 2003. Estimation of the activation energy of carbohydrate polymers blends as selection criteria for their use as wall material for spray-dried microcapsules. *Carbohydrate Polymers*. 53(2): 197-203. DOI:10.1016/S0144-8617(03)00052-3

Poncelet B., Poncelet D., Neufeld R. 1990. Preparation of hemolysate-filled hexamethylene sebacamide microcapsules with controlled diameter. *Canadian Journal of Chemical Engineering*. 68(3): 443-448. DOI: 10.1002/cjce.5450680314

Poncelet D., Lencki R., Beaulieu C., Halle J., Neufeld R., Fournier A. 1992. Production of alginate beads by emulsification/internal gelation. I. Methodology. *Applied Microbiology and Biotechnology*. 38(1): 39-45. DOI: 10.1007/BF00169416

Pszczola D. 1993. Gellan gum wins IFT's Food Technology Industrial Achievement Award. *Food Technology*. 47(9): 94-96

Robitaille R., Pariseau J., Leblond F. 1999. Studies on small (<350 µm) alginate-poly-L-lysine microcapsules. III. Biocompatibility of smaller versus standard microcapsules. *Journal of Biomedical Materials Research*. 44(1): 116-120. DOI: 10.1002/(SICI)1097-4636(199901)

Sanderson G., Bell V., Clark R., Ortega D. 1988. The texture of gellan gum. In: Phillips, G.O., Williams P.A. & Wedlock D.J. *Gums and stabilisers for the food industry 4*. Washington (USA). pp 219-229.

Sanderson, G. 1990. Gellan gum. In: Harris P. *Food gels*. New York: Elsevier science. pp 201-232.

Shahidi F., Han X. 1993. Encapsulation of Food Ingredient. *Critical Reviews in Food Science and Nutrition*. 33(6): 501-547

Stanton C., Gardiner G., Meehan H., Collins K., Fitzgerald G., Lynch B., Ross R.P. 2001. Market potential for probiotics. *American Journal of Clinical Nutrition*. 73(2): 476s-483s

Sun W., Griffiths W. 2000. Survival of bifidobacteria in yoghurt on gastric juice following immobilization in gellan-xanthan bead. *International Journal of Food Microbiology*. 61(1): 17-25. DOI:10.1016/S0168-1605(00)00327-5

Tang J., Tung M., Zeng Y. 1997. Gelling properties of gellan solutions containing monovalent and divalent cations. *Journal of Food Science*. 62(4): 688-712. DOI: 10.1111/j.1365-2621.1997.tb15436.x

Thies C. 1996. A survey of Microencapsulation processes. *Drugs and the pharmaceutical sciences*. 73(1): 1-19

Tyle P. 1993. Effect of size, shape and hardness of particles in suspension on oral texture and palatability. *Acta Psychologica*. 84(1): 111-118

Yáñez J., Salazar J. A, Chaires, L., Jiménez J., Márquez M., Ramos E. 2002. Aplicaciones biotecnológicas de la microencapsulación. *Avance y Perspectiva*. 21:313-319.

Yáñez J. 2007. Microencapsulación de *Lactobacillus spp*., empleando mezclas de biopolímeros de gomas arábiga, gelana y de semillas de mezquite, por polimerización interfacial. Doctoral thesis. Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional. México. pp 1-103.

Yáñez J., Ramos E.G., Salazar J. 2008. Rheological characterization of dispersions and emulsions used in the preparation of microcapsules obtained by interfacial polymerization containing *Lactobacillus sp*. *European Food Research Technology*. 226(5): 957-966. DOI: 10.1007/s00217-007-0617-9

Zhao R., Sun J., Torley P., Wang D., Niu S.2008. Measurement of particle diameter of *Lactobacillus acidophilus* microcapsule by spray drying and analysis on its microstructure. *World Journal of Microbiology Biotechnology and Biotechnology*. 24(8):1349-1354. DOI: 10.1007/s11274-007-9615-0