Development of alginate beads for probiotic encapsulation: influence of different parameters in the beads size. By P.E. Ramos[1], M. Muñiz-Alario[1,2], M.A. Cerqueira[1], A. Vicente and J.A. Teixeira[1], [1]*IBB — Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University do Minho, Campus de Gualtar, 4710-057 Braga, Portugal.* [2]*School of Industrial and Telecommunication Engineers, Department Chemical Engineering and Inorganic Chemistry, Universidad de Cantabria, 39005 Santander, Spain.* *Braga, Portugal, July 23, 2013.*

Abstract

In the present study different parameters were evaluated in the formation of alginate beads by ionotropic gelation (external extrusion). The size of alginate beads was determined and results showed that needle-$CaCl_2$ solution distance, flow rate, sodium alginate concentration, needle diameter and molecular weight of sodium alginate influenced alginate beads formation. Moreover, was concluded that needle diameter was the parameter that most influenced the beads mean size.

Introduction

Probiotics are a group of bacteria defined by the Food and Agriculture Association of the United Nations (FAO) and World Health Organisation (WHO) as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host's (1). Probiotics have been incorporated into food products such as: yoghurts, cheeses, ice cream, milk powders and frozen dairy desserts, bakery products (2). However, there are still several problems with respect to the low viability of probiotic bacteria in processed foods. A methodology that has been used to improve probiotic survival is encapsulation (3), that has been applied in the different bacteria and different food products, such as cheese (4,5), mayonnaise (6), cream (7). Several technologies can be applied to probiotic encapsulation and each of them provides microcapsules with different characteristics in terms of range size of particles and type of capsule. For example, emulsification allows the production of a wide particle size range from 0.2 to 5000 μm whereas, extrusion gives a smaller range size but it does not provide particles under 300 μm (8). Alginate is one of the most studied biopolymer as encapsulation material, due to its low cost, simplicity and biocompatibility. Alginate is an anionic copolymer of 1,4-linked-β-D-mannuronic acid and α-L-guluronic acid residues which forms a gel through the cross-linking of the guluronic acid blocks by the calcium ions, resulting in an a "egg-box" structure (9). The aim of this study was to evaluate the influence of production variables in alginate particles size.

Material and Methods

Materials - Lactococcus lactis cremoris (SK110/B697) (Culture collection from NIZO food research, The Netherlands) was inoculated into 200mL of M17 broth and incubated at 37oC for 24 h under anaerobic conditions. Sodium alginate (Protanal 8133, Protanal 8223 and Protanal LFR 5/60 Sample) was obtained from FMC BioPolymer. The alginate viscosity was measure with a 1% solution at 20 oC, at 30 rpm using a rotational viscometer.

Preparation of alginate-based beads by ionotropic gelation (external extrusion) - Sodium alginate and $CaCl_2$ solutions at different concentrations were prepared separately and autoclaved for 15 min at 121oC. Parameters such as flow rate, stirring speed, needle-$CaCl_2$ solution distance, alginate concentration, $CaCl_2$ concentration, needle diameter and alginate type were evaluated with the purpose of minimize the beads size and enhance the uniformity of the beads. The range considered for these variables was selected from previously published studies (10,11) and is presented below. In the first set of experiments, external variables of the system were studied in order to understand how the formation of alginate beads was affected. The following parameters were analysed: flow rate, needle-$CaCl_2$ solution distance and the stirring speed. At the beginning, 10 mL of sterile 2% (w/v) sodium alginate solution was extruded using a syringe and a pump with different flow rates, 1, 3 and 5 mL/min, through a 0.8 mm needle into sterile 0.1 M $CaCl_2$ (10 mL). The $CaCl_2$ solution was magnetically stirred at different rates, 60, 100 and 300 rpm, and the syringe was placed at 1, 5 and 10 cm above the $CaCl_2$ solution. Once the external variables affecting the system were determined, internal variables were studied. Variables such as the needle diameter (0.3; 0.6; 0.9 mm), alginate concentration (1, 2 and 3%) and $CaCl_2$ concentration (0.25; 0.5; 1 M) were evaluated. The flow rate of the pump was 1 mL/min and the volume of the extruded alginate was 10 mL. The beads, in this second experiment, were magnetically stirred at 300 rpm and the needle-$CaCl_2$ solution distance was 1 cm (parameters chosen based in results obtained in after the first set of experiments). In the third set of experiments, parameters such as alginate type (viscosity and M/G ratio), needle diameter (0.3; 0.6; 0.9 mm) and $CaCl_2$ concentration (0.25; 0.5; 1 M) was studied. For this purpose, 1% (w/v) sodium alginate solutions, using three different sodium alginates were extruded. The other parameters were kept constant and with the same values that in the second experiment. For the evaluation of studied variables on alginate beads formation, the diameters of 10 randomly selected beads, from each experiment, were photographed (Sony, 8MPx) and analysed using the software of public domain for the processing scientific picture Image J (Version 1.46r. ImageJ, National Institutes of Health, U.S.A.). A factorial design (Industrial DOE) was used for the study of variables affecting the beads size with a 2x3 factorial design with a central point. The standardized effects were carried out using Pareto's charts, considering $p < 0.05$ statistically significant. The software STATISTICA 7.0 (Statsoft, Inc., Tulsa, OK, USA) was used for all statistical analysis.

Results and Discussion

Experiment 1: Influence of the flow rate, needle-$CaCl_2$ solution distance and stirring rate. Considering the great number of experimental factors, a two-levels fractional factorial design with replications was used for screening purpose allowing the identification of 11 runs. Table 1 shows the different tested conditions, as well as, the mean diameter obtained for each experiment.

Table 1. Experimental design for experiment 1

Experiment	Flow rate (mL/min)	Needle-$CaCl_2$ solution distance (cm)	Stirring rate (rpm)	Size (mm) (Mean ± SD)
1	1	1	60	2.670 ± 0.186
2	1	10	60	3.028 ± 0.263
3	1	1	300	2.694 ± 0.100
4	1	10	300	2.683 ± 0.150
5	5	1	60	2.731 ± 0.121
6	5	10	60	2.863 ± 0.190
7	5	1	300	2.945 ± 0.084
8	5	10	300	2.912 ±0.210
9	3	5	100	2.755 ± 0.137
10	3	5	100	2.891 ± 0.124
11	3	5	100	2.879 ± 0.139

From results presented in table 1 it is possible to understand that smaller capsules were formed with lower flow rates and smaller needle-$CaCl_2$ solution distance, leading to the formation of beads with a size of 2.670 ± 0.186 mm. The smallest bead size was achieved at 60 rpm, and the stirring rate influence was not statistically significant (-0.18). Thus, 300 rpm, 1 mL/min and 1 cm of distance was used for the next experiments. In experiment 1, the variables that statistically influence the diameter mean size are needle-$CaCl_2$ solution distance (3.79) and flow rate (3.15).

Experiment 2: Influence of sodium alginate concentration, needle diameter and $CaCl_2$ solution concentration. Table 2 shows the conditions used for the different experiments, as well as, the mean size for the obtained beads. Results showed that the needle diameter influenced the size of the beads, where the smallest beads were obtained with 0.3 mm diameter needle (experiment 1, 2, 5 and 6) ranging from 1.910 ± 0.094 to 2.070 ± 0.125 mm. Considering alginate concentration, it was observed a less influence of this parameter on the size of the beads (2.73) and statistically the $CaCl_2$ concentration had no influence. The smallest beads present a mean size of 1.910 ± 0.094 mm, obtained for the following conditions: 3% (w/v) of alginate, 1 M of $CaCl_2$ and a needle of 0.3 mm diameter (experiment 6).

Table 2. Experimental design for experiment 2

Experiment	Alginate concentration (% w/v)	$CaCl_2$ concentration (M)	Needle diameter (mm)	Size (mm) (Mean ± SD)
1	1	0.25	0.3	2.168 ± 0.104
2	1	1	0.3	2.070 ± 0.125
3	1	0.25	0.9	2.595 ± 0.155
4	1	1	0.9	2.765 ± 0.122
5	3	0.25	0.3	2.070 ± 0.105
6	3	1	0.3	1.910 ± 0.094
7	3	0.25	0.9	2.909 ± 0.271
8	3	1	0.9	3.026 ± 0.225
9	2	0.5	0.6	2.387 ± 0.125
10	2	0.5	0.6	2.428 ± 0.097
11	2	0.5	0.6	2.483 ± 0.104

In experiment 2, the variables that statistically influence the diameter mean size are needle diameter (26.52) and alginate concentration (2.73). It was observed that beads were more spherical when using low alginate concentrations during the extrusion process (Figure 1). Table 2 shows the mean size of beads obtained with a alginate concentration of 3% (w/v), regardless the elongation formed. Therefore, in the third experiment, a 1% (w/v) sodium alginate solution was utilized in order to guarantee the uniformity and sphericity of the beads.

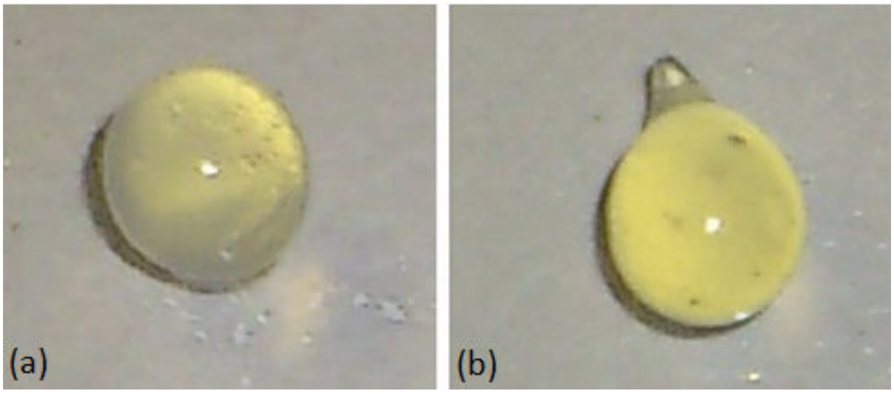

Fig. 1. Images of alginate beads (a) 1% (w/v) and (b) 3% (w/v)

Experiment 3: Influence of the alginate type, needle diameter and $CaCl_2$ solution concentration. In this case, three alginate types with different chemical characteristics were used. Table 3 shows the viscosity, ratio of mannuronic acid:guluronic acid (M/G ratio) and the molecular weight of alginates used.

Table 3. Properties of alginates used in experiment 3

Alginate type	Viscosity[a] (mPa·s)	M/G ratios[b]	Molecular weight (KDa) [b]
Protanal CR 8133	39	60-70/30-40	90-180
Protanal CR 8223	326	60-70/30-40	250 – 350
Protanal LFR5/60	7	25-35/65-75	20 – 60

Table 4. Experimental design for experiment 3

Experiment	Alginate type	Needle diameter (mm)	Concentration $CaCl_2$ (M)	Size (mm) (Mean ± SD)
1	CR 8133	0.3	0.25	2.474 ± 0.294
2	CR 8133	0.9	0.25	3.257 ± 0.319
3	CR 8133	0.3	1	2.244 ± 0.267
4	CR 8133	0.9	1	3.302 ± 0.284
5	LFR 5/60	0.3	0.25	2.970 ± 0.564
6	LFR 5/60	0.9	0.25	3.259 ± 0.483
7	LFR 5/60	0.3	1	2.767 ± 0.413
8	LFR 5/60	0.9	1	3.823 ± 0.535
9	CR 8233	0.6	0.5	2.335 ± 0.203
10	CR 8233	0.6	0.5	2.323 ± 0.128
11	CR 8233	0.6	0.5	2.516 ± 0.189

For CR 8133 alginate solution, the diameters of beads ranged from 2.244 ± 0.267 to 3.302 ± 0.284 mm (Experiment 1, 2, 3 and 4) (Table 4). For LFR 5/60 alginate solution, the size beads ranged between 2.767 ± 0.413 and 3.823 ± 0.535 (Experiment 5, 6, 7 and 8). The smallest size beads were 2.244 ± 0.267 mm corresponding to experiment 3. As showed in previous experiment, the needle diameter had a great influence in alginate beads size (Figure 1). The type of alginate used also influenced the formation of the beads, being the larger diameters obtained with LFR 5/60 alginate solution, whose molecular weight is the lowest. In experiment 3, variables that statistically influence the diameter mean size are needle diameter (10.63), alginate concentration (5.14) and $CaCl_2$ concentration (2.07). After understanding the factors that influence the size of the alginate beads and obtain the alginate bead with the lowest size (sample 6 of the experiment 2), the probiotic bacteria was encapsulated and achieved an encapsulation efficiencies higher than 99%.

Conclusion

Alginate beads formed by ionotropic gelation (external extrusion) were influenced by: needle-$CaCl_2$ solution distance, flow rate, sodium alginate concentration, needle diameter and molecular weight of sodium alginate. The smaller bead sizes were achieved with the lowest values of the previous parameters, with the exception of the molecular weight. The optimal size was 1.910 ± 0.094 mm, corresponding to 3% (w/v) of sodium alginate and 1 M $CaCl_2$. It was concluded that this method is not able to generate particles below these mean diameter sizes. The next step will be the formation of microparticles by internal extrusion.

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