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Production of alginate beads by emulsification/internal gelation. I. Methodology

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Summary. Small diameter alginate beads (microspheres) were formed via internal gelation of alginate solution emulsified within vegetable oil. Gelation was initiated by addition of an oil-soluble acid thereby reducing the pH of the alginate solution and releasing soluble Ca^{2+} from the citrate complex. Smooth, spherical, micron-sized beads were formed. The mean diameter ranged from 200 to 1000 μm, controlled by the reactor impeller design and rotational speed. The technique has potential for large-scale and continuous applications in immobilization.

Introduction

Early encapsulation methods involved the use of organic solvents or other reagents that were incompatible with many potential biological encapsulants (Chang et al. 1966). The use of various gel-forming proteins (collagen and gelatine) and polysaccharides (agar, calcium alginate, and carrageenan) resulted in milder, biocompatible immobilization techniques (Kennedy and Cabral 1983). The procedures generally involved heating the gel until liquefaction occurred (40–60° C), adding the immobilizant, then solidifying by cooling the solution. However, elevated temperatures may be incompatible with thermally labile material.

A more gentle and simple immobilization technique was developed, involving the addition of an ionic polysaccharide/immobilizant solution dropwise through a syringe needle into a solution of a divalent cation. The divalent ions cross-link the charged species on the polysaccharide, forming insoluble gel beads (Kierstan and Bucke 1977). Alginate is typically used as the ionic polysaccharide in conjunction with calcium ions as cross-linker for immobilizing many materials such as plant cells (Redenbaugh et al. 1986), mammalian cells (Lim and Sun 1980), yeasts (Shiotani and Yamane 1981), bacteria (Provost et al. 1985), insulin (Lim 1983), toners (Canon 1984), magnetite (Burns et al. 1985) or food products such as edible oils (Q. P. Corp. 1985). Beads of uniform size and quality are produced. However, reduction in bead size is limited by the syringe needle diameter and viscosity of the solution. As a result, beads less than 1 mm are difficult to produce. Smaller diameter beads facilitate mass transfer, enhancing fermentation performance and minimizing bead rupture due to gas formation and accumulation. Reduction in bead size has been attempted previously by air jets impinging on the needle (Miyawaki et al. 1980), electrostatic pulses (Hommel et al. 1988) or vibrating needles (Hulst et al. 1985).

For large beads (≈3 mm) formed by single droplet generation, the production rate may reach 24 l/h per needle (Hulst et al. 1985) and multiple needles permit small-scale industrial production (100 l, Moët & Chandon, France). However, the number of needles needed to maintain the flow rate is inversely proportional to the bead volume. Reducing the size to 500 μm or 100 μm requires the use of several hundreds or hundred thousands of needles operating concurrently: a complex and awkward solution (Dabora 1967). The most important limitation of the syringe-droplet technique is that it is not suited for industrial scale-up (Poncelet et al. 1992).

Bead formulation through emulsion techniques has only been applied on a few occasions with ionic polysaccharides. One adaptation involves a hot carrageenan/oil emulsion that is dropped into cold water (Lacroix et al. 1990). An oil-in-aqueous alginic acid emulsion can also be added dropwise to a $CaCl_2$ solution to encapsulate oil droplets in alginate (Lim and Sun 1980). The former technique involves elevated temperatures whereas the latter still involves single droplet generation. The difficulty of using emulsion techniques with ionic polysaccharide/$CaCl_2$ is that both reactants are insoluble in the oil phase.

An emulsion of a polysaccharide aqueous solution in oil can be added to a $CaCl_2$ solution, and thereby lead to

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bead formation. However, the particle size cannot be easily controlled and the capsules tend to coagulate into large masses before hardening properly. An internal gelation method was proposed whereby calcium ions are slowly liberated within the ionic polysaccharide via spontaneous breakdown of gluconolactone, resulting in acidification of an alginate slurry containing a calcium salt (Pelaez and Karel 1981). Molds of calcium alginate gel were cast, since it takes up to 5 min for the solution to harden. The gradual hardening would render the technique unsuitable for gelation within an emulsion due to aggregation of the beads.

In the present study, an alginate sol within an oil dispersion was gelled. Internal gelation was achieved through the rapid release of calcium ions from an insoluble citrate complex (Lencki et al. 1989). Calcium ion release was initiated via gentle acidification with an oil-soluble acid, which partitions to the aqueous alginate phase. By controlling the conditions under which the water-in-oil dispersion is produced, the bead diameter can be controlled within the range of 200 to 1000 μm. The low shear involved protects fragile encapsulants. Furthermore, since dispersions can be produced industrially in large equipment, the scale-up potential of this process is almost unlimited. Since toxic reagents or solvents are not used, biological and food applications may be considered.

Materials and methods

Reagents. Table 1 summarizes viscosity data for the different alginates used in this study. Canola oil was kindly provided by Canada Packers (Montréal, Canada). Surfactant span 80 (sorbitan triolate) was obtained from Sigma. Reagent grade calcium citrate, calcium chloride, sodium bicarbonate and acetic acid were purchased from Anachemia (Montréal, Canada).

Bead production. Sodium alginate was dissolved in a Waring blender or other high-shear mixing device for 2 min to yield a 1–4% alginate solution. Solutions were gently mixed for 1 h to facilitate deaeration. Buffer may be added to adjust the pH to the desired value, typically 8.0. Prior to use, the encapsulant was uniformly dispersed within the alginate (ratio up to 1:1). The alginate preparation was then mixed with a 20% slurry of calcium citrate in a proportion of 19:1.

The alginate-calcium citrate mixture containing encapsulant was dispersed for 15 min in Canola oil (1:6) with an impeller speed of 200–500 rpm. With continued stirring, a small additional volume of oil was added to the dispersion containing 0.1 to 1.0 ml glacial acetic acid for every 20 ml alginate to initiate gelification. Mixing was discontinued after 1–5 min. Beads were separated from the oil dispersion by partitioning into an equivalent volume of 50 mM $CaCl_2$ solution. The beads were then filtered on 30-μm sieves and washed with 1% Tween solution.

To compare the size distribution, alginate beads were prepared by dropping alginate solution into 50 mM $CaCl_2$. Small alginate drops were obtained by extruding the alginate solution through a needle subject to high electrostatic pulses (Hommel et al. 1988). Carrageenan/locust beam gum beads were prepared using an emulsion method described by Audet and Lacroix (1989).

Reactor configuration. The dispersion step was performed in either a flat or round-bottomed cylindrical reactor. The flat-bottomed reactor was used with or without four standard baffles. Reactors were designed to achieve equivalent liquid depth to diameter (Fig. 1) according to standard mixing protocols (Oldshue 1983; Rounsley 1983). Three types of impeller geometries were compared; six blade turbine, double flat blades of wire mesh, and a marine impeller. Illustrations and characteristic dimensions of the impellers and reactors are given in Fig. 1.

Bead size distributions and alginate viscosity. Volume size distributions of beads were obtained using a Malvern 2605-Lc particle size analyser. The alginate viscosity was measured at 20° C using a Brookfield viscometer model LVT with a small sample adaptor (model UL) for small volumes (16 ml).

Results

Figure 2 illustrates a typical size distribution for alginate beads formed by the process of dispersion/internal gelation. The size distribution curve consisted of two main peaks. Peak B represented up to 25% and peak A 65–95% of the total bead volume. Several smaller peaks (S) under 150 μm represented up to 10% of the bead volume.

Peak A was assumed to follow the normal law characterized by mean diameter and standard deviation on the basis that it is symmetrical, and generally represents more than 75% of the total bead volume. The significance of divergence from this simple model was estimated by the percentage of bead volume included in the

Table 1. Alginate characteristics

Supplier	Product	Guluronic content	Viscosity (cp)			
			1.0%[a]	1.2%[a]	1.8%[b]	3.0%[a]
Grinsted	Sobalg FD-120	Low	<50	—	100	—
Grinsted	Sobalg FD-124	Low	<50	—	158	—
Aldrich	18094-7	—	—	—	170	300
Sanofi	Satialgine S-170	Low	—	200	480	—
Grinsted	Sobalg FD-170	Low	300	—	1300	—
Sanofi	Satialgine SG-300	High	180	300	1464	—
Sanofi	Satialgine SG-500	High	300	500	1694	—
Sanofi	Satialgine S-550	Low	—	550	>2000	—

[a] From suppliers
[b] Brookfield viscometer, 0.3 rpm, $T = 20°$ C

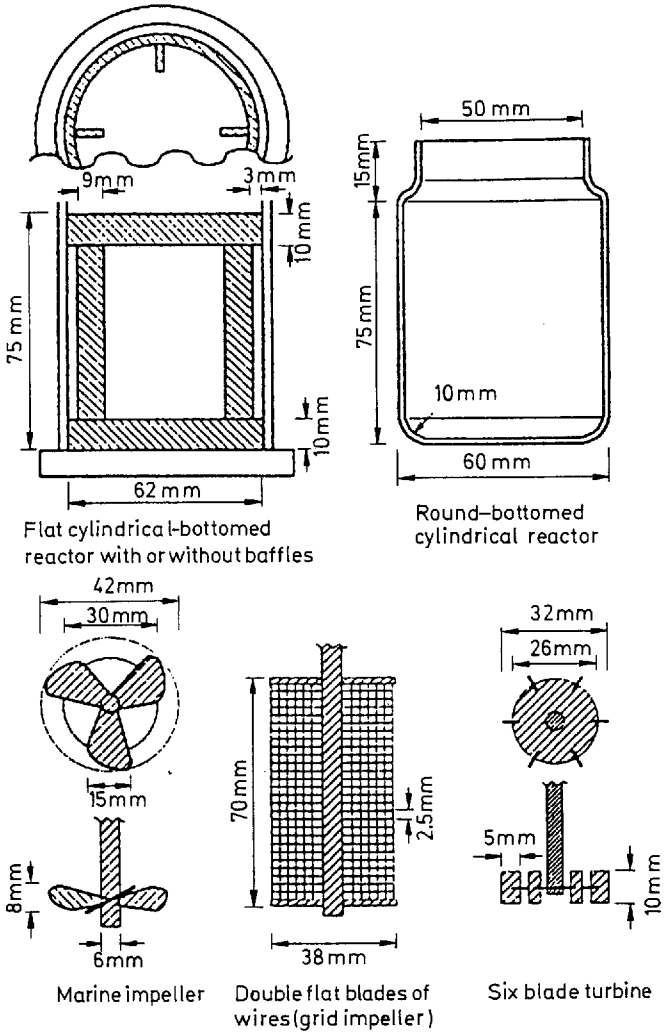

Fig. 1. Design of reactors and impellers

secondary peak B and the percentage of bead volume in the sum of the small peaks under 150 µm. The main peak was characterized by the mode (maximum frequency) and the diameter standard deviation, σ, obtained from the peak width at half peak height (Fig. 2). Reproducibility tests for the mean and standard deviation evaluation, summarized in Table 2, indicate a high degree of reproducibility in the measurements.

The size distribution of alginate beads obtained by emulsification/internal gelation was compared with the size distribution of alginate beads produced by extrusion under an electrostatic pulse. Standard deviations of the main peaks were 35 and 20% of the mean, respectively. In both cases, satellite peaks were observed representing up to 10% of the bead volume. Figure 3 also compares the size distribution of alginate and carrageenan beads, both obtained by emulsification. Satellite peaks were not observed with carrageenan beads.

The viscosity of the alginates varied widely with concentration and source of alginate, as shown in Table 1. The viscosity had little influence on the peak position in

Table 2. Reproducibility of size distribution parameter estimations

Sampling	Mean[a] (µm)	±SD (%)	σ[a] (µm)	±SD (%)
Batch to batch	392	15	39	8
Sample to sample (same batch)	355	10	38	10
Repetition on same sample	343	2	40	9

Data are based on three or more measurements using 1.8% Aldrich alginate, 2% Span and calcium citrate containing 50 µM Ca^{2+} in a round-bottomed reactor without baffles, stirred with a marine impeller at 400 rpm: σ, diameter standard deviation obtained from the peak width at half peak heights; ±SD, standard deviation

[a] For peak A

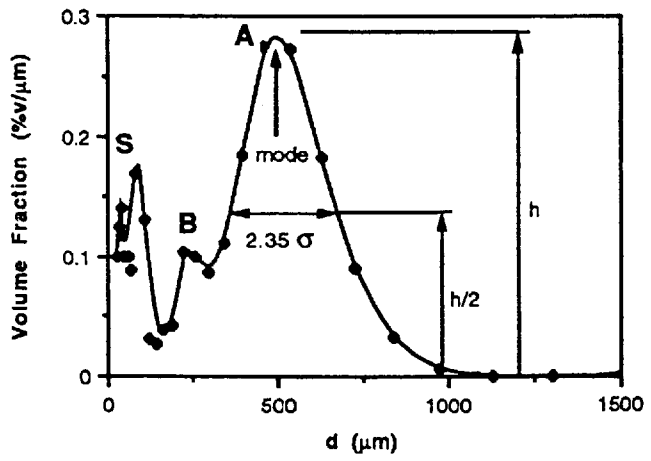

Fig. 2. Estimation of the size distribution parameters, using 1.8% Aldrich alginate, 2% Span 80, and calcium citrate containing 50 mM Ca^{2+} in a round-bottomed reactor without baffles stirred with a marine impeller at 400 rpm: h, peak height; h/2, half peak height; d, diameter of beads: σ, diameter standard deviation; S, B, A, peaks of bead sizes

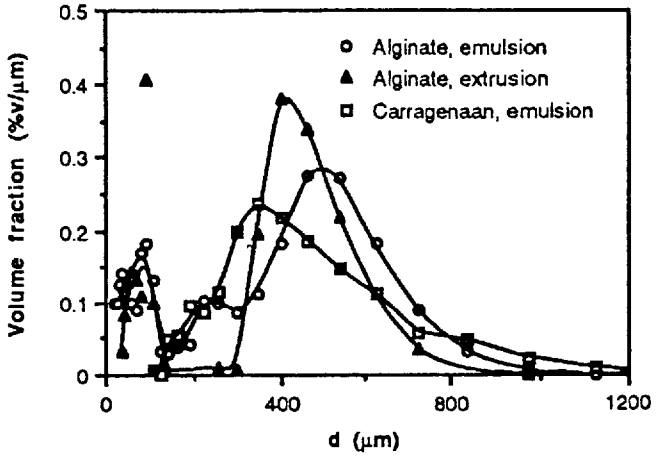

Fig. 3. Size distribution of different bead systems using the alginate emulsion (O) as in Fig. 2, alginate extrusion as following process described in Hommel et al. (1988), and 2% carrageenan (□), as in Fig. 2

Table 3. Impact of alginate viscosity and guluronic content on the size distribution of beads

Alginate	Viscosity (cp)	Guluronic content	Mode[a] (μm)	σ^a (%)	Peak A (% vol.)	Peak B (% vol.)
FD-170	100	Low	720	34	78	16
FD-120	158	Low	660	35	78	18
S-170	480	Low	690	35	78	20
FD-124	1300	Low	720	36	84	10
SG-300	1464	High	610	34	85	5
SG-500	1694	High	620	34	87	4
S-550	>2000	Low	670	29	77	16

Experiments were carried out in a round-bottomed reactor with calcium citrate containing 50 μM Ca^{2+}, 1.8% alginate and 2% Span 80, stirred with a marine impeller at 400 rpm
[a] For peak A

Table 4. Effect of the reactor and impeller design on bead size distribution

Impeller	Speed (rpm)	Mode[a] (μm)	σ^a (%)	Peak A (% vol.)
Grid with baffles	200	416	28	81
	300	312	34	86
	400	227	43	87
Grid without baffles	200	650	27	91
	300	338	35	87
	400	286	35	91
Turbine with baffles	200	1065	31	95
	300	431	33	90
	400	416	43	90
	500	208	42	92
Turbine without baffles	200	918	26	88
	300	615	39	87
	400	590	35	82
	500	281	31	77
Marine with baffles	200	1091	30	87
	300	615	37	85
	400	468	33	85
	500	406	34	88
Marine without baffles	200	1065	32	89
	300	688	34	82
	400	462	45	90
	500	389	34	88

Experiments were carried out in a cylindrical-bottomed reactor with 1.8% Aldrich alginate, 2% Span 80 and calcium citrate containing 50 μM Ca^{2+}
[a] For peak A

the size distribution, as seen in Table 3. An increase in the viscosity by a factor of 100 reduced the peak mode by less than 10%. However, the use of an alginate with high guluronic content (SG-300 and SG-500) favoured the formation of peak A. The use of high guluronic alginate resulted in a large peak A with a correspondingly small volume of beads in peak B (4%). Low guluronic alginate favoured peak B up to 20% of the total bead volume.

Dispersions of alginate in both vegetable and mineral oils were examined. Mineral oil resulted in larger bead diameters but hindered subsequent transfer of the beads

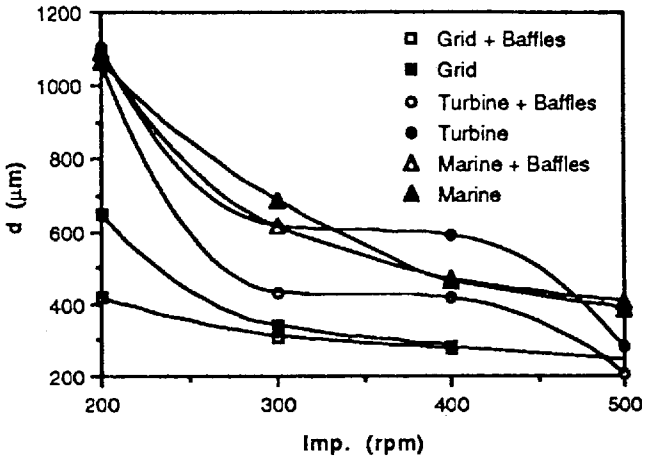

Fig. 4. Peak mode in relation to the impeller rotational speed (Imp.) for different impellers and reactor designs, using 1.8% Aldrich alginate, calcium citrate containing 50 mM of Ca^{2+}, and 2% Span 80

from the oil to the water phase. The use of vegetable oil was also prefered for food applications. The use of an emulsifier (0.5–8%) during the dispersion step did not significantly affect the size distribution but is needed to facilitate transfer of beads to the aqueous phase during separation.

Different reactor and impeller geometries were examined for alginate bead formation. Size and design parameters of the hardware are outlined in Fig. 1. As the alginate-in-oil dispersion was viscous, dead volumes in the reactor were occasionally observed. Alginate accumulated in these volumes forming aggregates, resulting in loss of yield. Baffle-to-wall and wall-to-bottom surface connections were the primary cause.

Varying the impeller rotational speed permitted control of the mean bead size (Fig. 4 and Table 4). However, both standard deviation (35 ± 5% of the mean) and volume fraction of the main peak (87 ± 4%) were not significantly affected by either the impeller speed or the reactor and impeller design (Table 4). The marine impeller provided a more homogeneous dispersion than the other impeller geometries. The grid mixers resulted in a lower mean size (Fig. 4). Grid dimensions of less than 2 mm did not promote alginate dispersion, but resulted in aggregate formation. Baffles appeared to influence bead size when using the turbine impeller or, at lower speeds, the grid-style mixer (Fig. 4).

Discussion

Alginate as cell entrapment material

Alginate has traditionally been the preferred immobilization material (Kierstan and Bucke 1977). More recently, other polysaccharides such as carrageenan (Audet and Lacroix 1989) and agarose (Nilsson et al. 1986) have been replacing alginate as a matrix for entrapment. Alginates are unstable in the presence of complexants such

as phosphate, due to loss of calcium. Carrageenan gels, while rheologically more stable than alginates, require high potassium levels (0.3 M) that are often incompatible, particularly in medical applications. At low concentrations of the cross-linker (Ca^{2+} for alginate and K^+ for carrageenan), alginate is stable for longer periods than carrageenan (unpublished data). Smidsrod and Skjak-Braek (1990) demonstrated that, by appropriate selection of alginate, beads were stable when suspended in solutions containing up to a 20 times higher concentration of sodium than calcium ions. Under similar conditions, carrageenan gels will liquefy. Producing beads from most other polysaccharides involves heating the sol to 40 or 50° C. The elevated temperatures are not compatible with some cell lines. These factors, combined with the ease of formulation, have resulted in alginate remaining the primary material for bead formation.

Often, gel beads are not efficient matrices for immobilization since cells are released from the gel (Champagne et al. 1991) and additional protection is needed in some applications against immunological reactions (Lim and Sun 1980). One solution is to coat the gel with another polymer, as is possible by contact with a polymer of opposite charge (e.g. poly-L-lysine on alginate) or through coating via precipitation (e.g. chitosan on agarose). In these cases, the mechanical resistance of the bead is less important than the resistance and permeability of the coating membrane.

While alginate remains an important immobilization matrix for the encapsulation of cells, the beads are normally formed by an extrusion method, yielding large beads (2–5 mm) resulting in mass transfer limitations and low bead production rates (ml/h) (Poncelet et al. 1992). The dispersion/internal gelation technique described in this report yields beads in a sub-millimetre size range, reducing mass transfer limitations (Neufeld et al. 1991), with the potential for formulation on an industrial scale (m^3/h). Studies of lactic acid bacteria encapsulation (not yet published) have shown that the proposed method is suitable for immobilisation of pure cell culture. Vegetable oil is heat sterilized and all operations are performed under aseptic conditions.

Size distribution and bead formation process

Most size determinations are conducted visually with the help of a microscope, counting a few to few hundred beads (Poncelet De Smet et al. 1989) or by sieving into a few size classes and weighing (Audet and Lacroix 1989). Automated instrumental analysis as used in the present study permits the counting of large numbers of beads (several thousand), distributed in a larger number of classes (32) over a significant diameter range (1 : 100), revealing small peaks and deviations that are often not visible in other analyses. The dispersion of aqueous sols in an oil phase may not achieve an equilibrium in the usual sense, as the final size distribution depends on processes that are nearly irreversible due to low rates of coalescence. High reproducibility of the shape of the size distribution curve and in the distribution parameters was observed in nylon bead formulations (Poncelet De Smet et al. 1990). In preparing alginate beads using an emulsification technique, mean and standard deviation values of the principal peak were less reproducible (15% compared to 8% for the nylon bead preparations) and the general shape of the size distribution curve was more variable. The analysis then focused primarily on the main peak.

Three types of peaks were generally observed; satellite peaks, secondary peak B and primary peak A. When a droplet breaks during emulsion formation, it is generally expected that two similar droplets will form. However, in keeping with the observation of stream break-up during bead formation by extrusion, it is more realistic to assume that a number of satellite drops will be formed at the same time [see fig. 29 of Grace (1982)]. Satellite bead formation is most likely the origin of the small peaks observed under 150 µm. Increases in turbulence may be the primary cause of satellite bead formation; thus reducing turbulence may minimize or eliminate satellite peaks.

Small peaks were also observed following formation of nylon and collodion-membrane-bound microcapsules using an emulsification technique, but to a lesser extent (1%) (Poncelet De Smet et al. 1989, 1990). In such systems, coalescence plays a more dominant role and it may be expected that equilibrium is attained after a short period of mixing. Due to the high gel viscosity in the case of polysaccharide bead formation, coalescence becomes less important, as small droplets will have less tendency to coalesce with larger droplets, and will contribute largely to the final size distribution.

Satellite peaks were not observed with carrageenan, but Audet and Lacroix (1989) demonstrated that small carrageenan beads were easily redissolved, and may not appear in the size distribution. Smaller beads are also more difficult to transfer from the organic phase and are easily lost during the washing step. This may also explain the lower contribution of satellite peaks to the carrageenan bead (Audet and Lacroix 1989) or microcapsule size distribution (Poncelet De Smet et al. 1989, 1990).

The simultaneous existence of peaks A and B seems to be related to alginate bead production. In contrast, microcapsules (Poncelet De Smet et al. 1989, 1990) or other gel beads (Audet and Lacroix 1989) result in a single main peak. Considering the main peak, the size dispersion for alginate microcapsules (Fig. 4) is similar to that of carrageenan beads. Nylon (Poncelet De Smet et al. 1990) and collodion beads (Poncelet De Smet et al. 1989) also resulted in a similar spread of size distribution. Most emulsification processes lead to standard deviations ranging from 30 to 40% of the mode (Haas 1987). To overcome this limitation, the extrusion method may be considered. The main peak generally results in a standard deviation of approximately 20% [Fig. 4 and Su et al. (1989)]. However, the present study demonstrates that satellite peaks also exist with such systems, resulting in larger global dispersion. The advantage with regards to the emulsification method is then largely reduced. Moreover, standard deviations increase when re-

ducing the bead size (Su et al. 1989) and under 500 μm the standard deviation is similar for both production systems.

Physico-chemical conditions

Generally size distribution in emulsions, and by consequence in beads and beads formed by an emulsification technique, are determined by the relationship between the surface tension and the dispersive forces (Hinze 1955). However, the alginate bead size distribution is independent of the presence or concentration of surfactant. For viscous solutions, it was proposed that the droplet size is controlled by the internal viscous resistance (Calabrese et al. 1986). Again, alginate molecular mass and concentration demonstrated only a minor effect on the size distribution. However, lowering the alginate concentration resulted in weakened beads that are easily broken. Grace (1982) observed that when the viscosity of the discontinuous phase increased, the viscous internal forces become the primary process, which opposes the dispersive forces. Increasing the viscosity leads to larger droplets. However, when the viscosity ratio between the discontinuous and continuous phase is larger than 3:4, which is generally the case in the present study, the droplets behave as rigid spheres. The usual laws of emulsification are then not entirely valid in the emulsification of polysaccharide solutions in oil.

For similar mixing conditions, the mean size of alginate beads was larger than that obtained for nylon or collodion microcapsules (Poncelet De Smet et al. 1989, 1990). The surface tension between the continuous phase and the dispersed phase are similar in microcapsule and alginate bead production. However, whereas the microcapsule size was affected by the emulsifier concentration (Poncelet De Smet et al. 1989, 1990), the mean alginate bead size remained constant. The mean size of alginate beads also did not appear to be affected by the initial alginate viscosity (less than 10%). However, when the alginate solution was mixed with calcium citrate, a rapid increase in viscosity was observed. Some of the calcium may be released in the medium even at pH 8.0, resulting in pregelification. The pregelled alginate would result in larger droplets (peak A). This process may not affect all droplets to the same extent. Some drops may contain larger crystals of calcium citrate or a lower concentration of calcium, leading to smaller droplets (peak B). Pregelification may mask the effect of the surfactant concentration and the initial alginate viscosity. Further investigations are then needed to understand the droplet breakage processes and the physicochemistry of calcium release to adequately control both the mean size and the size distribution of alginate beads.

Reactor design

Peak dispersion is not strongly affected by the reactor and impeller design (Table 4). However, it has a strong influence on the yield and the mean diameter (Fig. 4). During development of the dispersion, alginate demonstrated a tendency to accumulate in stagnant regions of the reactor. The simplest reactor design avoiding corners minimized this problem. Baffles had little influence on either the dispersion or the mean diameter, thus offered little benefit.

As observed previously (Poncelet De Smet et al. 1989, 1990), grid mixers resulted in a more homogeneous dispersion of the mixing energy throughout the reactor volume, resulting in smaller diameter beads. However, adhesion of alginate on the grid resulted in a lower yield. With both marine and grid-style mixers, the mean diameter decreased on increasing the impeller speed. The influence of the impeller speed is more complex with a turbine. Introduction of baffles seems to have little impact on the size except at moderate speed.

Conclusion

The dispersion/internal gelation procedure outlined in the present study illustrates a new and innovative technique for producing small ionic polysaccharide microspheres. This method is fast, easily scaled, and uses biocompatible materials. Potential applications are found in biotechnology, medicine and the food industry. The greatest limitation of the emulsification methods is that they result in a large dispersion of bead diameters. Selection of appropriate calcium vectors, alginate and conditions that favour coalescence may reduce the dispersion. The relationship between alginate structure and both the size distribution and bead strength may offer the best compromise in alginate selection. Another approach to reducing the size dispersion is to select a reactor configuration with low turbulence and homogeneous shear. Low turbulence will reduce the formation of satellite droplets whereas homogeneous shear may decrease the spread of the main peak. Presently, other types of reactor design are being tested for continuous dispersion formation, which provides a homogeneous and low shear. In addition, emulsification is achieved in a very short time, reducing the possibility of the pregelification of the alginate.

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