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CALCIUM-ALGINATE BEADS AS CARRIERS FOR BIOCATALYST ENCAPSULATION

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ABSTRACT

Beads resulting from the gelation of sodium alginate (SG800® and S1100®) with calcium chloride were evaluated in function of reagent concentrations ($CaCl_2$: 0.05M, 0.1M and 0.4M; sodium alginate: 5 g/L, 10 g/L and 20 g/L) and temperatures (30°C and 40°C). Temperature does not affect significantly the production of beads, differently from reagent concentrations. The beads presented a high resistance to mechanical attrition (agitation of 1,000 rpm for 1 h) and fermentation stress (represented by the joint action of carbon dioxide pressure and the increasing in cell number). A linear correlation between the number of beads obtained and the calcium and alginate consumed, as well as an encapsulation yield over 90% (for example, SG800: 20g/L; 0.4M $CaCl_2$; 98% and S1100: 20g/L; 0.4M $CaCl_2$; 97%), was also observed.

KEYWORDS: Immobilization, encapsulation, calcium chloride, alginate, entrapment.

INTRODUCTION

Immobilization is a technique in which a biocatalyst (enzyme, cells, and organelles) is associated to an insoluble and inert material (called carrier or support). The interaction between the biocatalyst and the carrier can be by binding or physical retention. The immobilization by binding involves two methods, i.e., binding to carriers – through covalent bonding, ionic interaction or adsorption –, and cross-linking (including the co-cross-linking variant, in which a bi-functional reagent such as glutaraldehyde could be used). The physical retention method comprises matrix entrapment (beads or fiber pellets) and membrane

enclosure (encapsulation and membrane reactor). Table 1 shows some advantages and disadvantages of immobilization.

Among all types of immobilization methods, the encapsulation of biocatalysts in calcium alginate beads deserves special attention because it is a soft method in the sense that the biocatalyst (commonly an enzyme) does not suffer any significant injury at the level of molecular structure mainly due to the absence of an enzyme-carrier interaction. The molecules of enzyme are surrounded by an insoluble gelatinous and semipermeable Ca^{2+}-membrane, which is simultaneously permeable and impermeable to low and high MW molecules, respectively. This characteristic, on one hand, limits its applicability to enzymes that require low MW substrates; in the other hand, it can entrap any kind of organelles or cells without limitation. In addition, the reaction between sodium alginate and calcium solution occurs in aqueous medium at room temperature (25^oC – 40^oC), pH (4.5 – 6.5), and low stirring (20 – 100 rpm).[2]

Table 1: Advantages and disadvantages of biocatalyst immobilization.[1]

Advantages	Disadvantages
Use of continuous processes	Decreasing catalytic activity
Reutilization	Biocatalyst-carrier random interaction
Increasing of the thermal stability	Inexistence of a general immobilization method
Catalyst absence in the product and effluent	Diffusion limitations imposed by the carrier; alteration of enzyme molecule structure
Diversification of the use of enzymes	The type of immobilization chosen might consider the characteristic of the process to be used
Use of different types of reactors	High cost of the pure biocatalyst when covalent bonding or cross-linking methods are used

Alginates are a group of polysaccharides (molecular weight ranging between 33,000 and 400,000 g/mol) extracted from the cell walls of brown algae (*Macrocystis pyrifera, Laminaria hyperborean* and *Ascophyllum nodosum*). Alginates are linear polymers consisting of 1,4-linked β-D-mannuronic acid (M) and 1,4 α-L-guluronic acid (G) residues arranged in an homogeneous (poly-M, poly-G) or heterogeneous (MG) block-like patterns.[3] Their main physical-chemical properties – viscosity, sol/gel transition (jelly capability in presence of divalent cation) and water-uptake ability – are related to the amount and distribution pattern of M-blocks and G-blocks along the polymer backbone. Gelling properties are strongly associated to the M/G ratio. In addition, alginate gels with a M/G < 1 are stiffer, brittler and

mechanically more stable than M/G >1 gels. Moreover, alginate characterized by a high proportion of M-blocks forms gradually softer and more elastic gels.

Available alginates – mainly as alginic acid, sodium alginate, ammonium alginate, calcium alginate, and propylene glycol alginate - have a large spectrum of applications such as in food industry (emulsifier, texturizer, stabilizer, thickener, formulation aid, firming agent, flavor adjuvant, surfactant, and humectants)[4], in pharmaceutical industry (color diluents, emulsifier, film former, humectants, diluents in capsule formulation, tablet binder and disintegrant, sustained release and release-modifying agent, taste masking agent, thickener, suspending and viscosity increasing agent, and stabilizer)[5][6][7][8], and in chemical-pharmaceutical industry (alginate is used mainly to make beads for entrapping enzymes, cells and organelles). Alginates can also be used in a large variety of pharmaceutical products because they are regarded as biocompatible, non-immunogenic and nontoxic materials.[9] The alginate solution/gel transition can occur under physiological conditions, for example, in the presence of divalent cations and in an acidic environment of body fluids. Thereby, nonwoven dressings of calcium alginate capable of exchanging ions with wound exudates have been used for the treatment of infected surgical wounds and as epidermis fistulae healing aids.[10][11] An exciting field for alginate application, in a near future, is in tissue engineering, which would allow confectioning 3D-microencapsulated pancreatic islets to treat type 1diabetes, for instance.[12][13]

Encapsulation of enzymes or cells in alginate beads is advantageous for the chemical-pharmaceutical industry because it is a low cost carrier, widely available in the market, and sold by numerous suppliers.[11] In addition, the alginate is easily handled, requiring soft reaction conditions for gelling in presence of divalent cations and low-tech apparatus for beads confection (Figure 1).[1]

Among the several biocatalysts useful in the chemical-pharmaceutical industry, undoubtedly, *Saccharomyces cerevisiae* cells have a great relevance for ethanol fermentation.[14][15] Thereby, this biocatalyst was used as example for encapsulation purposes.

This work deals with the gelation of two types of commercial alginates - one M/G <1 (SG800) and the other M/G >1 (S1100) – at different concentrations (5 g/L, 10 g/L and 20 g/L) in the presence of $CaCl_2$ solution at concentration of 0.05M, 0.1M or 0.4M for

producing calcium-alginate beads. Moreover, methods for controlling the number of beads produced and for evaluating their mechanical resistance were also discussed.

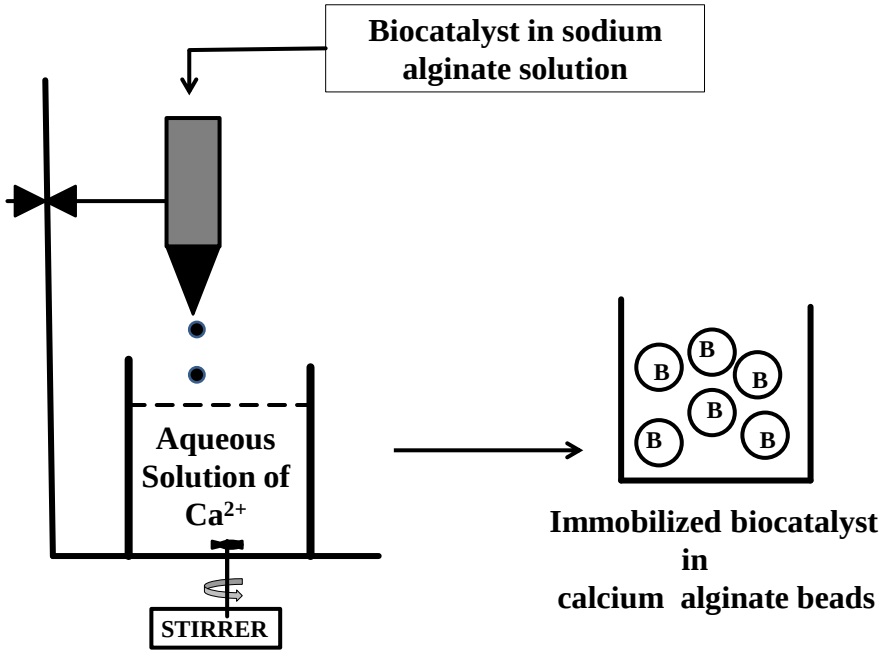

Figure 1: Sketch of encapsulation of a biocatalyst (enzyme, organelle or microbial cells) in calcium alginate beads.

MATERIAL AND METHODS

MATERIAL

Sodium alginates [SATIALGINE® forms: SG800 (M/G=0.5; 400-490cP) and S1100 (M/G=1.2; 550-750cP)] were purchased from Sanofi Bio-Industries (Paris, France). All other reagents were of analytical grade. *Saccharomyces cerevisiae* was grown as described elsewhere.[15]

METHODS

Encapsulation

The alginate solution was prepared by dissolving 5 g, 10 g or 20 g of SG800 or S1100 in 1L of deionized water. Then, the solution was left to rest at 4°C for 24 h. After, 30 mL of the alginate solution was dropped into a 100mL solution of 0.05M, 0.1M or 0.4M $CaCl_2$ from a cylindrical reservoir (inner diameter = 14 mm; height = 34 mm). The outlet extremity (internal diameter = 2 mm) was positioned 80 mm from the calcium chloride solution surface. The gelation was carried out under a stirring of 50 rpm at 30°C or 40°C and pH 6.5-7.0. Then,

the beads (mean diameter = 4 mm) were separated using a sieve, and the residual Ca^{2+} was titrated with 0.1M EDTA in the presence of murexide. The amount of calcium consumed was correlated with the number of beads obtained.

The yeast cells were encapsulated in calcium alginate beads by dropping 30 mL of alginate aqueous suspension (5.0, 10.0 or 20.0 g/L of SG800 or S1100) containing yeast cells (around $1x10^5$ cells/mL) into 100 mL of 0.05M, 0.1M or 0.4M $CaCl_2$ solution buffered at pH 6.5. The beads formed were left to harden in the calcium solution for 24 h. Then, the beads were separated using a sieve, and the residual $CaCl_2$ solution was collected. After immobilization, the beads retained on the sieve, the recipient containing the sodium alginate cell suspension, and the dropping device were washed with deionized water. The washing water and the residual solution of calcium were mixed to determine the number of cells in suspension.

The percent of yeast cells encapsulated (YCE) was calculated as follows:

$$YCE = (CCWW \div ICC).100 \qquad \text{(Eq. 1)}$$

Where CCWW = Cell concentration in washing water (cells/mL); ICC = Initial cell concentration (cells/mL).

Mechanical attrition

In a 600-mL Becker containing 300 mL of distilled water, five hundred beads were suspended. The suspension was submitted to agitation of 1,000 rpm for 1 h. After, the cracked beads, if present, were detected using an optical microscope.

Cell fermentation

Six hundred beads containing entrapped cells (overall concentration of $0.8x10^5$ cells/mL) were aseptically added into a 500-mL round glass flask containing a magnetic stirrer and 250 mL of culture medium - clarified and sterilized blackstrap molasses (pH 4.6) containing 30g/L of total reducing sugars (TRS) supplemented with $(NH_4)_2SO_4$ (5.1 g/L), $Na_2HPO_4.12H_2O$ (2.4 g/L) and $MgSO_4.7H_2O$ (0.075 g/L). The flask was hermetically closed with a lid containing an adapted thermometer and a U-shaped glass tube (the external extremity was immersed into 30 mL of 0.5M sodium dithionite). The flask was enveloped with a heating jacket, and placed on the plate of a magnetic disk (Figure 2). The fermentation was carried out at 37°C, initial pH 4.6, and stirring of 200 rpm for 24 h. After that, the beads were separated by filtration using a sieve. The filtrate was collected for analytical

determinations (ethanol, residual total reducing sugars and final pH). The beads recovered were examined using an optical microscope in order to verify signs of cracking. After, they were dissolved into 0.5M sodium hexametaphosphate to count the total number of cells formed.

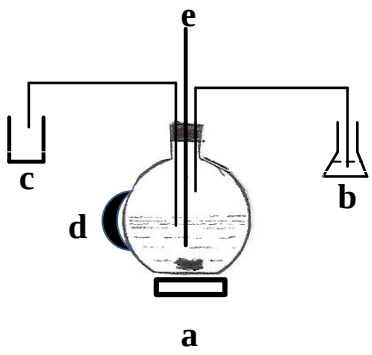

Figure 2: Sketch of the bench fermenter used for evaluating the fermentative capability of yeast cells. (a) Magnetic stirrer; (b) Flask containing the 0.5M sodium dithionite; (c) Sample harvester; (d) Heating jacket; (e) Thermometer.

Analytical Methods

Titration of Ca^{2+} with EDTA

After separating the calcium-alginate beads, the residual $CaCl_2$ solution was collected. The remaining Ca^{2+} was measured. Twenty milliliters of this solution were titrated with 0.1M EDTA solution in the presence of murexide as indicator. The titration was carried out at 30°C and pH 12.0. One milliliter of 0.1M EDTA is equivalent to 4 mg of Ca^{2+}.

Measurement of total reducing sugars

The total reducing sugars (TRS) were measured using a spectrophotometer as described elsewhere.[16]

The absorbance (read at 540 nm) was converted into RS, expressed as glucose, through a standard curve (Eq. 2). A standard glucose solution (0.2 mg/mL) was used, from which 0.2-1.0 mL aliquots were taken.

$Y_{gluc} = 2.63.X_{gluc} + 0.027$ (r = 0.996) (Eq. 2)

Where Y_{gluc} = absorbance and X_{gluc} = amount of glucose (mg).

Ethanol measurement[1]

An aliquot of 1 mL of the fermented medium free of cells was distilled for 5 min. The distillate was collected in a 250-mL conical flask containing 20.0 mL of 0.2M $K_2Cr_2O_7$ (dissolved in 32% (w/w) H_2SO_4 [d= 1.84 g/cm^3]). The flask was left in a water bath at 70^{o}C for 20 min. After cooling, the excess of $K_2Cr_2O_7$ was titrated with 0.35M $Fe(NH_4)_2SO_4.6H_2O$ (dissolved in 2% (w/w) of H_2SO_4 [d = 1.84 g/cm^3]) in the presence of o-phenantroline as indicator. A blank was carried out using 20.0 mL of distilled water instead of the fermented medium. The ethanol concentration, expressed as g/L, was calculated as follows:

$$E = 11.5.V.M'.\ [1- (V_a \div V_b)] \qquad \text{(Eq. 3)}$$

Where V = Volume of potassium dichromate solution (mL); M' = Molar concentration of potassium dichromate; V_a = Volume of ferrous sulphate consumed (mL); V_b = Volume of ferrous sulphate consumed in the blank test (mL).

Cell counting[1]

The cell counting was carried out using a conventional Neubauer chamber (area = 1/400 mm^2; height = 0.100 mm). The cell concentration (X_{cell}), expressed as number of cells/mm^3, was calculated as follows:

$$X_{cell} = [(A.D) \div 0.4] \qquad \text{(Eq. 4)}$$

Where A = Number of cells counted; D = Dilution of cell suspension; Chamber volume = 0.4 mm^3.

RESULTS AND DISCUSSION

Firstly, the data related to the confection of beads by dropping a sodium alginate solution (SG800 or S1100) on 0.05M, 0.1M or 0.4M $CaCl_2$ were disposed in tables such as Table 2. The calculation of the Ca^{2+} consumed, through equation 5, was presented in the Appendix. Plotting alginate (or number of beads formed) versus Ca^{2+} consumption presented in Table 2 resulted in straight lines, as showed in Figures 3 and 4.

$$m_C = 4.(Y_0 - Y) \qquad \text{(Eq. 5)}$$

Where m_C = mass of Ca^{2+} consumed (mg); Y_0 = volume of 0.1M EDTA solution consumed before gelation (mL); Y = volume of 0.1M EDTA solution consumed after gelation (mL).

As the sodium alginates used (SG800: 400-490cP and S1100: 550-750cP) have different viscosities, then the effects of temperature (30°C or 40°C) on the formation of beads could be significant (Figures 5 and 6).

Table 2: Amount of SG800 and Ca^{2+} consumed in function of the number of beads obtained. Test conditions: 30°C; pH 6.5; 100 rpm; 5g/L of SG800; and 15 mL of 0.05M $CaCl_2$ were titrated with 0.1M EDTA.

Beads (number)	SG800 (mL)	SG800 (mg)	EDTA (mL)	Ca^{2+} (mg)	
				Residual	Consumed
0	0	0	7.5	30	0
100	5.0	25	7.4	29.6	0.4
200	11.8	59	7.2	28.8	1.2
300	18.0	90	6.9	27.6	2.4
400	25.5	127.5	6.6	26.4	3.6
500	33.4	167	6.1	24.4	5.6

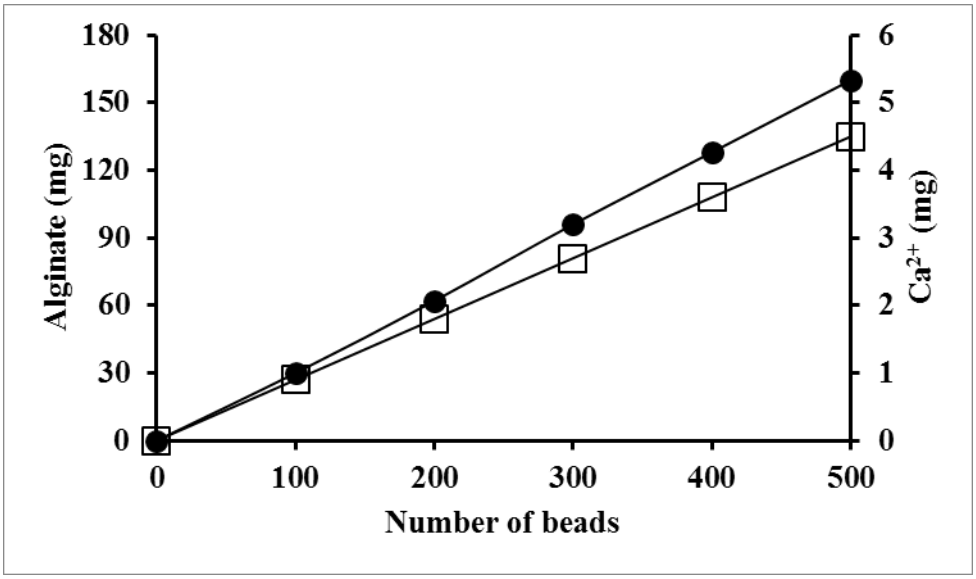

Figure 3: Variation of sodium alginate (●) and Ca^{2+} (□) consumed in function of the number of beads obtained. The gelation was carried out with SG800 (5 g/L) and 0.05M $CaCl_2$ at 30°C.

The minimum square linear regression equations related to Figure 3 are:

$Y_{alg} = 0.3223.X - 1.24$ (r = 0.9998) (Eq. 6)

$Y_{Ca} = 0.009.X$ (r = 1) (Eq. 7)

Where Y_{alg} = amount of sodium alginate consumed (mg); Y_{Ca} = amount of Ca^{2+} consumed (mg); X = number of beads.

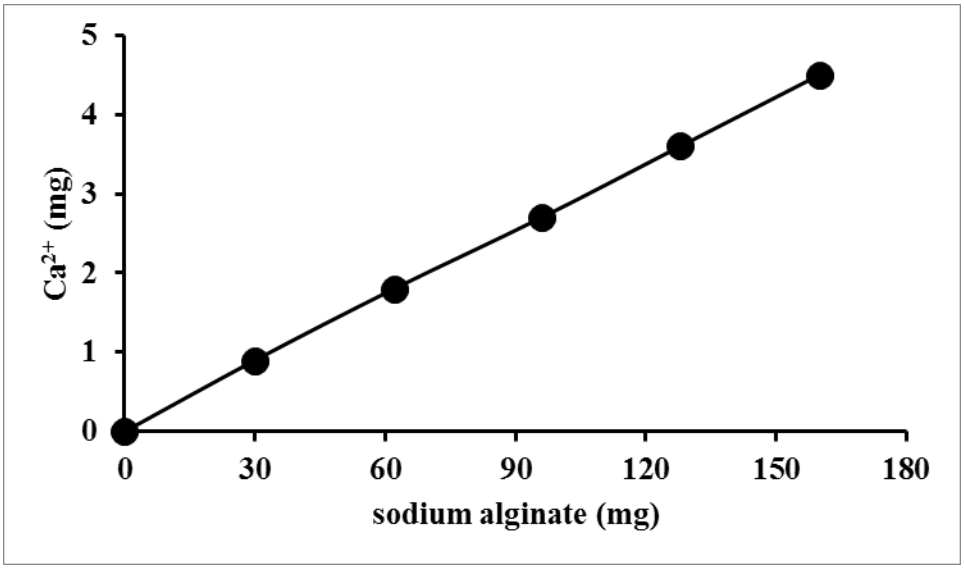

Figure 4: Correlation between sodium alginate and Ca^{2+} consumed. The gelation was carried out with SG800 (5 g/L) and 0.05M $CaCl_2$ at 30°C.

The minimum square linear regression equation related to Figure 4 is:

$Y_{Ca} = 0.0279.X_{alg} + 0.0351$ (r = 0.9997) (Eq. 8)

Where Y_{Ca} = amount of Ca^{2+} consumed (mg); X_{alg} = amount of sodium alginate consumed (mg).

As the amounts of alginate and Ca^{2+} consumed are linearly correlated either between them (Figure 4) or to the number of beads obtained (Figure 3), the variation of Ca^{2+} will henceforth be set against the number of beads obtained for evaluating the effects of sodium alginate and Ca^{2+} concentrations on the alginate-Ca^{2+} gelation.

As SG800 and S1100 have different viscosities, the temperature could probably affect the calcium-alginate gelation for bead production. In order to verify this, beads were obtained through the gelation of SG800 or S1100 solution (10 g/L) with 0.1M or 0.4M $CaCl_2$ at 30°C and 40°C (Figures 5 and 6).

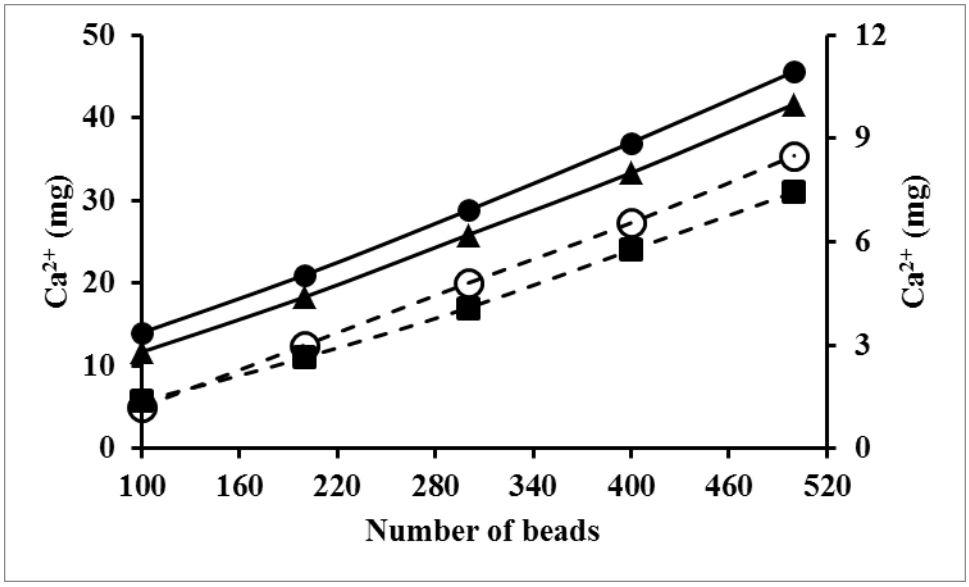

Figure 5: Number of beads in function of Ca^{2+} consumed for SG800 (10 g/L) polymerized at 30°C (dashed lines) and 40°C (solid lines) with $CaCl_2$ 0.1M [30°C: (o); 40°C: (▲)] and 0.4M [30°C: (■); 40°C: (●)].

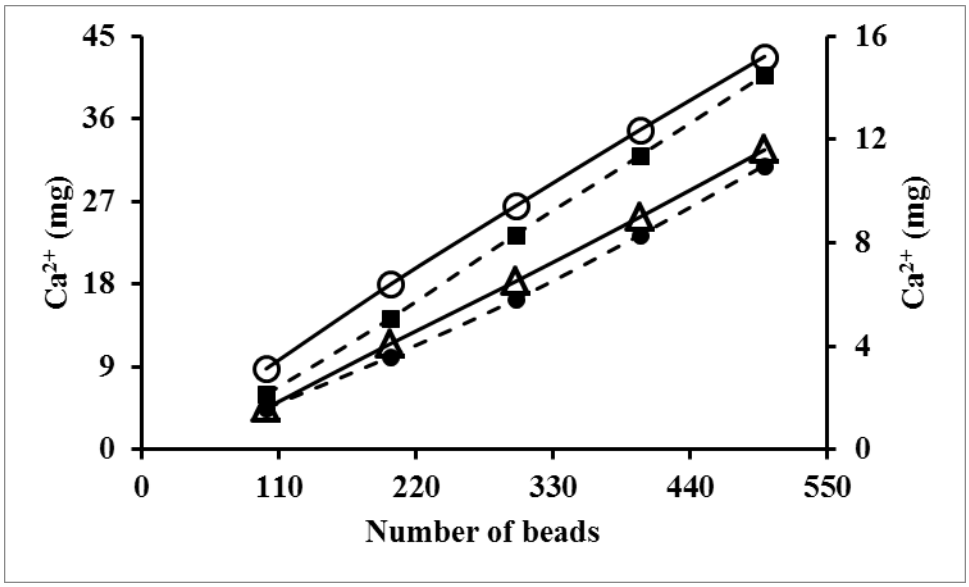

Figure 6: Number of beads in function of Ca^{2+} consumed for S1100 (10 g/L) polymerized at 30°C (dashed lines) and 40°C (solid lines) with $CaCl_2$ 0.1M [30°C: (●); 40°C: (Δ)] and 0.4M [30°C: (■); 40°C: (o)].

The minimum square linear regression of the straight lines presented in Figures 5 and 6 are:

$*Y_{0.1M} = 0.0182.X_{SG800} - 0.635$ (r = 0.9998) (Eq. 9)

$*Y_{0.4M} = 0.0651.X_{SG800} - 1.34$ (r = 0.9996) (Eq. 10)

$**Y_{0.1M} = 0.0180.X_{SG800} + 0.88$ (r = 0.9992) (Eq. 11)

$** Y_{0.4M} = 0.0690.X_{SG800} + 5.52$ (r = 0.9993) (Eq. 12)

* $Y_{0.1M} = 0.0235.X_{S1100} - 0.99$ (r = 0.9997) (Eq. 13)

* $Y_{0.4M} = 0.0873.X_{S1100} - 2.89$ (r = 0.9993) (Eq. 14)

** $Y_{0.1M} = 0.0249.X_{S1100} - 0.91$ (r = 0.9991) (Eq. 15)

** $Y_{0.4M} = 0.0848.X_{S1100} + 0.74$ (r = 0.9996) (Eq. 16)

Where ($*Y_{0.1M}$; $*Y_{0.4M}$) and ($**Y_{0.1M}$; $**Y_{0.4M}$) are the amount (mg) of Ca^{2+} consumed in the gelation of alginates (SG800 and S1100) with 0.1M or 0.4M $CaCl_2$ solution at 30°C and 40°C, respectively. X_{SG800} and X_{S1100} are the number of beads obtained using SG800 and S1100, respectively.

The angular coefficients of equations 9-16 were disposed in Table 3.

Table 3: Variation of the angular coefficients of equations 9-16 regarding temperature (30°C and 40°C) and concentration of $CaCl_2$ solution (0.1M and 0.4M).

ALGINATE (10 g/L)	$CaCl_2$ (M)	TEMPERATURE		VARIATION (%)
		30°C	40°C	
SG800	0.1	0.0182	0.0180	**1.1**
	0.4	0.0651	0.0690	**5.7**
VARIATION (%)		**72.0**	**73.9**	
S1100	0.1	0.0235	0.0249	**5.6**
	0.4	0.0873	0.0848	**2.9**
VARIATION (%)		**73.1**	**70.6**	

Table 3 shows that the Ca^{2+}-alginate gelation was less affected by temperature (variation lower than 6%) than by $CaCl_2$ concentration (variation higher than 70%). Probably, the effects of low temperature on the gelation could be due to the narrow interval used (from 30°C to 40°C). Using a temperature higher than 40°C could be dangerous mainly when enzymes are encapsulated because the thermal instability of enzymes is well known.[17] Thereby, the gelation temperature is set at 30°C. Regarding the concentration of $CaCl_2$ solution, it is reasonable to suppose that more Ca^{2+} available means that more could be inserted into the lattice of the calcium-alginate bead envelopes, which have been visualized as having an "egg-box" format.[18]

Table 4: Minimum square linear regression equations regarding the variation of Ca^{2+} consumed (Y) in function of the number of beads obtained (X). The calcium-alginate gelation was carried out at 30°C.

Type	(g/L)*	$CaCl_2$ (M)	Equation	r	$^aR_1$	$^bR_2$	$^cR_3$
SG800	5	0.4	Y = 0.0777X + 0.305	0.9995	1	1.10	1.02
		0.1	Y = 0.0275X + 0.181	0.998	2.83	1	1
		0.05	Y = 0.009X	1	8.63	1.41	2.21
	10	0.4	Y = 0.0636X – 1.34	0.998	1	1.37	1.24
		0.1	Y = 0.0182X – 0.635	0.9998	3.49	1.29	1.51
		0.05	Y = 0.0110 – 0.0714	0.9996	5.78	1.02	1.81
	20	0.4	Y = 0.0789X – 0.381	0.9998	1	1.10	1
		0.1	Y = 0.0243X – 0.0381	0.9991	3.25	1.12	1.13
		0.05	Y = 0.0199X + 0.0857	0.9997	3.96	1	1
S1100	5	0.4	Y = 0.0858X – 0.124	0.9998	1	1	1.02
		0.1	Y = 0.0264X – 0.0333	0.9998	3.25	1.04	1.03
		0.05	Y = 0.0127X + 0.114	0.9992	6.76	1	1.53
	10	0.4	Y = 0.0873X – 2.89	0.9993	1	1	1
		0.1	Y = 0.0235X – 0.99	0.998	3.71	1	1.15
		0.05	Y = 0.01125 – 0.145	0.997	7.76	1	1.72
	20	0.4	Y = 0.0869X + 0.752	0.9995	1	1	1.01
		0.1	Y = 0.0271X + 0.0381	0.9997	3.21	1	1
		0.05	Y = 0.0194X + 0.100	0.9990	4.48	1.03	1

*Alginate (SG800 and S1100) concentration; $^aR_1$ = ratio between line inclinations for different calcium chloride concentrations; $^bR_2$ = ratio between line inclinations for different types of sodium alginate; $^cR_3$ = ratio between line inclinations for different sodium alginate concentrations.

Table 4 shows the effects of alginate types (SG800 and S1100), concentrations of calcium chloride (0.05M, 0.1M and 0.4M) and sodium alginate (5 g/L, 10 g/L and 20 g/L) on the production of beads. It can be seen that $CaCl_2$ and sodium alginate concentrations affect the Ca^{2+}-alginate interaction, which, in turn, increases as the sodium alginate concentration decreases: R_1 equal to 8.63 and 7.76, respectively, for SG800 (5 g/L) and S1100 (10 g/L). These results agree with those described elsewhere.[19][20] Moreover, the proportional variation of the calcium consumed and the number of beads obtained could be used as a method for attaining any desired number of beads. This method can be considered simpler, quicker and cheaper than the others found in the literature.[19][20][21]

Table 5: Fermentative capability of yeast cells encapsulated in calcium-alginate beads. The resistance of the beads to mechanical attrition (MA) and the yield of cell encapsulated (YCE) are also presented.

Type	Alginate (g/L)	$CaCl_2$ (M)	[a]Nx10^{-7} (cell/mL)	[b]TRS (g/L)	[c]E (g/L)	[d]pH	[e]MA	YCE (%)
SG800	5	0.4	5.768	2.0	0.68	5.5	-	89
		0.1	4.996	2.2	0.44	5.4	-	80
		0.05	*5.068	3.0	0.46	5.5	-	65
	10	0.4	5.358	3.4	1.1	5.3	+	78
		0.1	5.282	3.5	2.7	5.1	+	94
		0.05	*4.987	3.2	2.3	5.0	+	79
	20	0.4	5.780	2.6	1.4	5.5	+	98
		0.1	5.182	2.8	1.5	5.4	+	95
		0.05	5.347	3.4	0.46	5.6	+	83
S1100	5	0.4	5.569	2.7	2.9	5.6	-	90
		0.1	4.998	3.0	0.50	5.7	-	76
		0.05	5.107	2.9	1.8	5.1	-	48
	10	0.4	4.296	3.2	2.9	5.4	+	90
		0.1	4.995	2.8	2.6	5.8	+	95
		0.05	*4.657	3.4	2.2	5.0	+	83
	20	0.4	4.798	2.8	2.0	5.0	+	97
		0.1	5.236	3.2	1.7	5.1	+	89
		0.05	*5.687	2.0	2.6	5.3	+	71

*Observed bead cracking; [a]Total number of cells within beads after fermentation; [b]Total reducing sugars consumed (TRS); [c]Ethanol formed; [d]pH at the end of fermentation; [e]Mechanical Attrition [beads cracked (-); beads not cracked (+)].

Table 5 shows that the beads resisted well to mechanical attrition, except for those produced with SG800 and S1100 (5g/L). Moreover, the internal stress suffered by the beads due to the increase in the number of cells plus the pressure promoted by the carbon dioxide generated by fermentation led to envelope cracking of only four types of beads, i.e., those made with S1100 (10g/L; 0.05M $CaCl_2$), S1100 (20g/L; 0.05M $CaCl_2$), SG800 (10g/L; 0.05M $CaCl_2$) and SG800 (5g/L; 0.05M $CaCl_2$). This result is remarkable insofar as the internal pressure promoted only by carbon dioxide ranged from 2.67 atm to 4.67 atm (see Appendix). Finally, yields of cell encapsulation over 90% were observed with SG800 [(10g/L; 0.1M $CaCl_2$) and (20g/L; 0.1M or 0.4M $CaCl_2$]; S1100 [(10g/L; 0.1M $CaCl_2$) and (20g/L; 0.4M $CaCl_2$).

CONCLUSIONS

The data presented allow concluding that there is a linear relation between the calcium and sodium alginate consumed and the number of beads produced. Therefore, this method could

be useful for obtaining any number of beads. Temperature (30°C and 40°C) exerts no significant role on beads production, differently from calcium chloride and sodium alginate concentrations, which changed, respectively, within the ranges 0.05M-0.4M and 5 g/L-20 g/L. Most bead types resisted to mechanical attrition or internal stress (a combination of increasing in cell number and carbon dioxide pressure). Yields of cell encapsulation over 90% were observed in beads made of SG800 [(10 g/L; 0.1M $CaCl_2$) and (20 g/L; 0.1M or 0.4M $CaCl_2$]; S1100 [(10 g/L; 0.1M $CaCl_2$) and (20 g/L; 0.4M $CaCl_2$).

APPENDIX

Calculation of residual Ca^{2+}

In a 0.1M EDTA solution, there is 0.1 mol of EDTA in 1,000 mL of solvent (water in the present case). In a volume different from 1,000 mL, the amount of mol is given by the following equation:

$X = (0.1Y)/1000$ (Eq. A1)

Where X = mol of EDTA consumed; Y = volume of 0.1M EDTA consumed (mL).

The mol of Ca^{2+} can be written as

$x = m/M = m/40$ (Eq. A2)

Where x = mol of Ca^{2+}; m = mass of Ca^{2+} (g); M = ion-gram of Ca^{2+}.

Considering the stoichiometry of EDTA-Ca^{2+} reaction as 1:1, then

$m/40 = 0.1Y/1000$ (Eq. A3)

or $m_R = 4Y$ (Eq. A4)

Where m_R = residual mass of Ca^{2+} (mg).

Before gelation: $m_R = m_{Ro} = 4Y_0$ (Eq. A5)

Where Y_0 = volume of 0.1M EDTA consumed before gelation (mL); m_{Ro} = residual mass of Ca^{2+} at Y_0.

After gelation: $m_C = (m_{Ro} - m_R)$ (Eq. A6)

Where m_C = mass of Ca^{2+} consumed (mg).

Substituting the equations A4 and A5 in A6, the result is

$m_C = 4.(Y_0 - Y)$ (Eq. A7)

For instance, Y_0 = 7.5 mL and Y = 6.9 mL (Table 2). Then, equation A7 is:

$m_C = 4.(7.5 – 6.9) = 2.4$ mg.

Estimation of bead internal pressure due to CO_2

The volume of one bead (radius (Ra) = 2 mm) can be calculated by the equation:

$V = (4/3).\pi.(Ra)^3$ (Eq. A8)

Therefore, one bead has a volume of 33.51 mm^3 or $33.51x10^{-6}$ L.

Considering the Clapeyron's equation:

$p.V = n.R.T$ (Eq. A9)

Where p = pressure (atm); V = volume (L); n = mol of CO_2; T= absolute temperature (^oK); R = 0.08205 (L.atm/mol.^oK).

As the total number of beads used was 600 and the fermentation was carried out at 310^oK (37^oC), equation A9 can be written as follows:

$p = (n \times 310 \times 0.08205)/(600 \times 33.51x10^{-6}) = 1265n$ (Eq. A10)

Assuming that 100g of glucose provide 46.4g of CO_2[22], then 0.20g and 0.35g of glucose (Table 5) provide 0.0928g (0.00211 mol) and 0.1624g (0.003691 mol) of CO_2, respectively. Substituting these values in the equation A10, then the internal pressure (p) was equal to 2.67 atm and 4.67 atm, respectively.

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REFERENCES

1. Rocha-Filho JA, Vitolo M. Guide for Practical Classes on Biotechnology of Enzymes and Fermentation. São Paulo; Edgard Blücher, 2017.
2. Arruda LMO, Vitolo, M. Characterization of invertase entrapped into calcium alginate beads. Applied Biochemistry and Biotechnology, 1999; 81: 23-33.
3. Remminghorst U, Rehm BHA. Bacterial alginates: from biosynthesis to applications. Biotechnology Letters, 2006; 28: 1701-1712.
4. Cable CG. Sodium Alginate. In: Handbook of Pharmaceutical Excipients, London; Pharmaceutical Press, 2009; 622-24.

5. Repka MA, Singh A. Alginic acid. In: Handbook of Pharmaceutical Excipients, London; Pharmaceutical Press, 2009; 20-22.
6. Shah SA, Thassu D. Ammonium alginate. In: Handbook of Pharmaceutical Excipients, London; Pharmaceutical Press, 2009; 41.
7. Gable CG. Calcium alginate. In: Handbook of Pharmaceutical Excipients, London; Pharmaceutical Press, 2009; 83-85.
8. Nause RG, Reddy RD, Soh JLP. Propylene glycol alginate. In: Handbook of Pharmaceutical Excipients, London; Pharmaceutical Press, 2009; 594-95.
9. Sachan KN, Pushkar S, Jha A, Bhattcharya A. Sodium alginate: the wonder polymer for controlled drug delivery. Journal of Pharmacy Research, 2009; 2(8): 1191-99.
10. Wahl EA, Fierro TR, Peavy TR. In vitro evaluation of scaffolds for the delivery of mesenchymal stem cells to wounds. BioMed Research International, 2015; 2015: Article ID 108571.
11. Szekalska M, Pucilowska A, Szymanska E, Ciosek P, Winnicka K. Alginate: current use and future perspectives in pharmaceutical and biomedical applications. 2016; http://dx.doi.org/10.1155/2016/7697031.
12. Calafiore R, Basta G. Clinical application of microencapsulated islets: actual prospective on progress and chalanges. Advanced Drug Delivery Reviews, 2014; 67: 84-92.
13. Aljohani WJ, Wenchoo L, Ullah MW, Zhang X, Yang G. Application of sodium alginate hydrogel. Journal of Biotechnology and Biochemistry, 2017; 3(3): 19-31.
14. Vitolo M. Effect of saline solution and osmotic pressure on invertase extraction from cell wall of baker's yeast. European Journal of Pharmaceutical and Medical Research, 2019; 6(5): 158-163.
15. Vitolo, M. Invertase activity of intact *Saccharomyces cerevisiae* cells cultured in sugarcane molasses by batch fermentation process. World Journal of Pharmacy and Pharmaceutical Sciences, 2019; 8(3): 126-137.
16. Vitolo, M. Autolysis of baker's yeast and partial purification of invertase in the presence of surfactants. European Journal of Pharmaceutical and Medical Research, 2019; 6(5): 84-90.
17. De Queiroz AAA, Vitolo M, De Oliveira RC, Higa OZ. Invertase immobilization onto a radiation, induced graft copolymerized polyethylene pellets. Radiation Physics and Chemistry, 1996; 47: 873-80.
18. Smidsrod O, Skjak BG. Alginate as immobilization matrix for cells. Trends in Biotechnology, 1990; 8: 71-8.

19. Ogbonna JC, Amano Y, Nakamura K. Elucidation of optimum conditions for immobilization of viable cells by using calcium alginate. Journal of Fermentation Bioengineering, 1989; 67: 92-6.
20. Martinsen A, Skajak BG, Smidsrod O. Alginate as immobilization material: correlation between chemical and physical properties of alginate gel beads. Biotechnology Bioengineering, 1989; 33: 79-89.
21. Ogbonna JC, Pham CB, Matsumara M, Kataoka H. Evaluation of some gelling agents for immobilization of aerobic microbial cells in alginate and carragenan gel beads. Biotechnology Techniques, 1989; 3(6): 421-24.
22. Blanch HW, Clark DS. Biochemical Engineering. New York; Marcel Dekker, 1996.