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ENCAPSULATION OF INVERTASE IN BARIUM ALGINATE BEADS

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ABSTRACT

Invertase (E.C. 3.2.1.26) was encapsulated in barium alginate beads. The gelation was carried out at 30°C by stirring (360 rpm and 540 rpm) using alginate concentrations (5 g/L and 10 g/L), barium nitrate concentrations (0.1M and 0.2M), and three pH of alginate-invertase solution (4.0, 6.7 and 8.0). Invertase encapsulation yielded 75.2% and encapsulated invertase activity yielded 0.249 U/mL. These results were obtained using beads produced with 10 g/L alginate (SG800) and 0.1M barium nitrate at pH 4.0, 30°C and 540 rpm. The kinetic constants for soluble and encapsulated invertase, calculated using Hanes-Woolf's plot, were K_M = 52.9 mM, V_{max} = 0.368 U/mL, $(K_M)_{encap}$ = 7.51 mM and $(V_{max})_{encap}$ = 0.269 U/mL. The beads were reused three times batchwise without significant loss of invertase activity.

KEYWORDS: Immobilization, invertase, alginate, encapsulation, entrapment.

INTRODUCTION

Encapsulation is a type of immobilization in which a biocatalyst (enzyme, cells, and organelles) is involved by a semipermeable membrane that allows low MW molecules to cross it freely.[1] This is a gentle method because gelation between sodium alginate and barium ion occurs under smooth aqueous conditions (temperature: 25°C – 40°C, pH: 4.5 – 6.5, and stirring: 20 – 100 rpm). Thus, the enzyme does not suffer any significant injuries at the molecular level mainly due to the absence of physicochemical interaction between the enzyme and the carrier. For example, by encapsulating invertase (an enzyme with a tertiary structure level) in alginate beads, there is an improvement of the catalytic activity due to the extemporaneous aggregation of invertase molecules in tetramers (a quaternary structural level).[2] In addition, barium alginate beads can be produced using a low-tech apparatus and

the sodium alginate is a cheap commodity plentifully available in the market and sold by several suppliers.[1][3]

Alginates are extracted mainly from algae species such as *Macrocystis pyrifera*, *Laminaria hyperborean* and *Ascophyllum nodosum*.

Alginates are a group of linear polysaccharides (molecular weight ranging between 33,000 and 400,000 g/mol) consisting of homogeneous or heterogeneous arrangements of 1,4-linked β-D-mannuronic acid (M) and 1,4 α-L-guluronic acid (G) residues. Their main physicochemical properties – viscosity, sol/gel transition (jelly capability in the presence of divalent cations) and water-uptake ability – are related to the amount and distribution pattern of M-blocks and G-blocks along the polymer backbone.[1][3] The sol/gel transition property, which occurs at room temperature, allows attaining a diversity of semisolid or solid structures under mild conditions. Such structures have a large variety of industrial applications.

Alginates (alginic acid, sodium alginate, ammonium alginate, calcium alginate, and propylene glycol alginate) have a large spectrum of applications in food industry (as emulsifier, texturizer, stabilizer, thickener, among others), in pharmaceutical industry (as emulsifier, film former, humectants, tablet binder and disintegrant, for instance), and in chemical-pharmaceutical industry (as carrier for entrapping enzymes, cells and organelles).[1][2][4] Alginates are labeled as biocompatible, non-immunogenic and nontoxic compounds.[5] Thereby, they can be used in association with calcium or barium ions (calcium alginates) in nonwoven dressings for the treatment of infected surgical wounds and as an aid in epidermis fistulae healing.[6][3] Moreover, the alginate is becoming a reference material in tissue engineering, envisaging the confection of 3D-microencapsulated human cells – for instance, microencapsulation of pancreatic islets to treat type 1 diabetes.[7]

Sodium alginate (certainly one of the most widely investigated alginates in the pharmaceutical and biomedical field) is water-soluble and forms stable viscous solutions provided the pH of the solvent is above 3.6 and the ionic strength is low. Sodium alginate gelation can be induced in the presence of divalent ions (such as Ca^{2+} or Ba^{2+}) or by lowering the solvent pH below the pKa of the alginate using lactones such as d-glucano--lactone. The former procedure is useful for attaining alginate beads – applied to entrap enzymes, cells, drugs and organelles-, whereas the latter is useful for preparing sheet pellicles for therapeutic purposes.

Alginate beads for applying in bioreactors must be spherical, resistant to shear force and simultaneously permeable for low MW substances and impermeable for macromolecules. Beads with these properties can be attained through a combination of factors such as concentration of alginate and divalent cation, solvent pH, temperature, and agitation. Of course, the number of combinations involving the cited factors would be practically limitless. In the case of Ba^{2+}-alginate, it has been noted that beads meeting the three characteristics cited could be obtained using alginate and barium concentrations, respectively, at the intervals 5-10 g/L and 0.1-0.2M. Additionally, the solvent pH between 4.0 and 8.0, agitation up to 540 rpm, and temperature of 30^{o}C also contributed to the confection of suitable beads. Alginate concentration below 5 g/L and/or barium concentration below 0.1M produce non-spherical beads.

Among the several biocatalysts useful in the chemical-pharmaceutical industry, invertase (E.C.3.2.1.26) has undoubtedly a great use in sugar hydrolysis. Invertase – one of the first enzymes to be identified (by Berthelot in 1860) and produced in large scale (since the beginning of the last century) – has been largely used in industry (mainly in sucrose hydrolysis) and more recently as medicine and reagent.[8][9]

This work studies the invertase entrapped in barium alginate beads aiming their use in sucrose hydrolysis. The effects of alginate concentration (5 g/L and 10 g/L) and type (SG800: M/G < 1; S1100: M/G > 1), pH of alginate solution (4.0, 6.7 and 8.0), agitation (360 rpm and 540 rpm) and barium nitrate concentration (0.1M and 0.2M) on the encapsulation of invertase were studied. In addition, the soluble and encapsulated invertase activities were determined, as well as their kinetic parameters (K_M and V_{max}). Reusability of encapsulated invertase was also evaluated.

MATERIAL AND METHODS

Material

Sodium alginates [SATIALGINE® forms: SG800 (M/G=0.5; 400-490cP; granules ≤ 200μ; and humidity ≤ 15%) and S1100 (M/G=1.2; 550-750cP; granules ≤ 160μ; and humidity ≤ 15%)] were purchased from Sanofi Bio-Industries (Paris, France). Invertase (Invertin®), with a protein concentration of 2.2 mg/mL, was purchased from Merck (Gernsheim, Germany). All other reagents were of analytical grade.

METHODS

Encapsulation

The alginate solution was prepared by dissolving 5 g or 10 g of SG800 or S1100 in 1 L of deionized water (pH adjusted to 4.0, 6.7 or 8.0). Then, the solution was left resting at 4°C for 24 h. Two milliliters of Invertin® and 18 mL of the alginate solution were mixed, then dropped into a 100-mL solution of 0.1M or 0.2M $Ba(NO_3)_2$ from a cylindrical reservoir (inner diameter = 14 mm and height = 34 mm). The outlet extremity (internal diameter = 2 mm) was positioned 80 mm from the surface of barium nitrate solution. The gelation was carried out under agitation of 360 or 540 rpm at 30°C. Then, the beads (mean diameter = 3 mm) were left to harden for 12 h in the barium solution. Finally, the beads were separated through a sieve and washed with 20 mL of deionized water. Each batch of gelation led to 500 beads.

The yield of invertase encapsulated (YIE) was evaluated by the equation:

$$YIE = [(V_{encap}) \div (V_{sol})] \times 100 \qquad \text{(Eq. 1)}$$

Where (V_{encap}) = Activity of immobilized invertase (mg RS/min.mL); (V_{sol}) = Activity of soluble invertase (mg RS/min.mL); RS = Reducing sugars.

Invertase Activity Measurement

Soluble invertase

Two hundred milliliters of sucrose solution (100 g/L in 0.01M acetate buffer, pH 4.6) and 1 mL of Invertin® were added to an Erlenmeyer flask. The hydrolysis was carried out for 60 min at 35°C and agitation of 450 rpm. Aliquots between 0.1 mL and 0.3 mL were taken at every ten min to monitor the reaction. The aliquots were transferred to Folin-Wu test tubes containing 1.0 mL of alkaline Somogy's solution. The tubes were immersed in a boiling water bath for ten min. Thereafter, the procedure was followed as described elsewhere.[10]

The initial invertase activity (V_{sol}) was calculated (always in triplicate) using the slopes of reducing sugars (RS) *versus* reaction time plots (Figure 1 is an example of such a plot). One invertase unit (U) was defined as the quantity of RS (milligrams) formed per minute under the conditions of the test. The soluble invertase had an activity equal to 0.368 ± 0.0015 U/mL of reaction medium.

Immobilized invertase

Five hundred barium-alginate invertase beads were suspended in 50 mL of deionized water and left stirring for 1 h. After that, the beads were separated through a sieve and the washing water was collected for soluble protein detection. Then, the beads were suspended in 200 mL of buffered sucrose solution, and the reaction was carried out as described above. The initial encapsulated invertase activity (V_{encap}) was calculated (always in triplicate) using the slopes of reducing sugars (RS) *versus* reaction time plots (Figure 1). In all tests, the amount of protein washed out from the beads was lower than 5 μg/mL. The encapsulated invertase had an activity varying from 0.148 to 0.249 U/mL and from 0.140 to 0.218 U/mL, respectively, for SG800 and S1100.

ANALYTICAL METHODS

Titration of Ba^{2+} with EDTA

After separation of the barium-alginate beads, the residual $Ba(NO_3)_2$ solution was collected. The remaining Ba^{2+} was then measured. Twenty milliliters of this solution was 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 equal to 13.7mg of Ba^{2+}.

Soluble protein measurement

Protein was determined based on the difference between UV absorbance measured at 215 and 225 nm using bovine serum albumin (BSA; Fraction V) as a standard. By using a 0.1 mg/mL (w/v) of BSA solution, the linear correlation between ΔAbs ($Abs_{215nm} - Abs_{225nm}$) and protein concentration (P) (varying from 10 to 100 μg/mL) was:

$$\Delta Abs = 5.90x10^{-3}P - 7.0x10^{-4} \quad (r = 0.9997) \quad (Eq.\ 2)$$

Reducing sugars measurement

The reducing sugars (RS) were measured by spectrophotometer as described elsewhere.[11]

The absorbance (read at 540nm) was converted into RS, expressed as glucose, through a standard curve (Eq. 3). A standard glucose solution (0.2 mg/mL) was used, from which 0.2-1.0 mL aliquots were taken.

$$Y_{gluc} = 2.63.X_{gluc} + 0.027 \quad (r = 0.996) \quad (Eq.\ 3)$$

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

RESULTS AND DISCUSSION

The amount of Ba^{2+} consumed in function of the number of beads obtained was plotted as shown in Figure 1. The minimal square linear regression equations are:

$Y = 0.0250X_{S1100} + 0.198$ (r = 0.9993) (Eq. 4)

$Y = 0.0194X_{SG800} + 0.148$ (r = 0.9996) (Eq.5)

Where Y = Ba^{2+} consumed (mg); X_{S1100} and X_{SG800} = Number of beads related to alginates S1100 and SG800, respectively.

The linear correlation between these variables was observed in all kinds of beads confectioned - SG800 or S1100 (5 g/L or 10 g/L) dropped in barium nitrate solution (0.1M or 0.2M) at a pH of 4.0, 6.7 or 8.0 and agitation of 360 rpm or 540 rpm. This result corroborates that observed in a previous work that produced Ca^{2+}-alginate beads.[1]

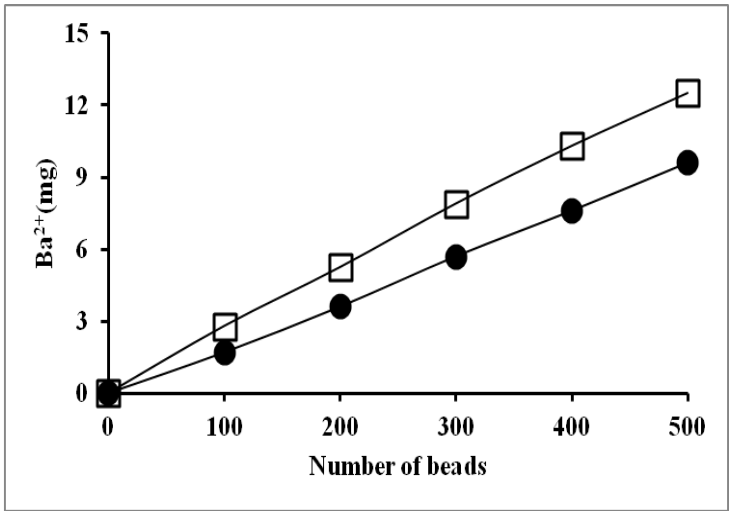

Figure 1: Number of beads in function of the Ba^{2+} consumed. The gelation conditions were 10 g/L alginate [SG800 (●); S1100 (□)], 0.1M $Ba(NO_3)_2$, pH = 6.7, agitation: 360 rpm and 30°C.

Figure 2 shows the formation of reducing sugars (Y) in function of reaction time (X) for soluble and encapsulated invertase. The minimal square linear regression equations are:

$Y_{sol} = 0.331\ X + 0.518$ (r = 0.998) (Eq. 6)

$Y_{SG800} = 0.242\ X + 0.281$ (r = 0.992) (Eq. 7)

$Y_{S1100} = 0.152\ X + 0.341$ (r = 0.997) (Eq. 8)

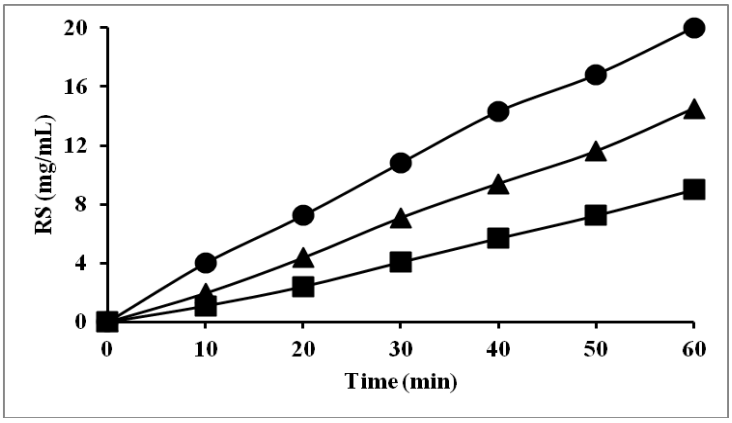

Figure 2: Formation of reducing sugars in function of the reaction time catalyzed by soluble invertase (●) and Ba^{2+}-alginate invertase [SG800 (▲) and S1100 (■)]. The beads were obtained by jellifying 10 g/L of alginate with 0.2M $Ba(NO_3)_2$ at pH 8.0, 360 rpm and 30°C.

The slope of each straight line represents the activity for soluble (0.331 U/mL) and encapsulated invertase - Ba^{2+}-SG800 (0.242 U/mL) and invertase-Ba^{2+}-S1100 (0.152 U/mL).

By applying equation 1, the yield of invertase encapsulated (YIE) in beads of Ba^{2+}-SG800 and Ba^{2+}-S1100 - obtained under the gelation conditions cited in the caption of Figure 2 was 73.1% and 45.9%, respectively. YIE values for other gelation conditions are presented in Tables 1 and 2.

The invertase encapsulation yield (YIE) and the bead invertase activity varied according to the gelation conditions (pH, stirring, barium and alginate concentration) used during the production of beads (Tables 1 and 2).

The YIE varied from 44.7% to 75.2% for SG800 (M/G <1) and from 42.3% to 55.9% for S1100 (M/G >1), whereas the bead invertase activity varied between 0.148 U/mL and 0.249 U/mL for SG800 and between 0.143 U/mL and 0.218 U/mL for S1100. The most significant fact relates to the types of alginates (SG800 and S1100), which differ in α-L-guluronic acid content (SG800 > S1100). Probably, the high amount of this monomer leads to a major number of structures with an "egg-box" format along the polysaccharide chain, which, in turn, can accommodate the barium ions more efficiently.[3] In addition, the pH ≥ 4.0 guarantees a net negative charge of alginate molecules (pKa: 3.38 - 3.65), making them more avid for chelating Ba^{2+}. Furthermore, at pH 4.0, the invertase molecules do not have electrical charge ("Zwitterion" form; pHi = 4.0), which, in turn, could facilitate their encapsulation

inside the beads, zeroing any possibility of chemical interaction with the barium-alginate carrier. This, associated with an adequate stirring (540 rpm) and balanced reagent concentrations (SG800 = 10 g/L and $Ba(NO_3)_2$ = 0.2M), led to the highest YIE (75.2%) and invertase activity (0.249 U/mL).

By setting YIE ≥ 70%, only the beads produced with SG800 have surpassed that limit (5 g/L, 0.1M, pH 8.0 and 540 rpm; 10 g/L, 0.1M, pH 4.0 and 540 rpm; 10 g/L, 0.1M, pH 8.0 and 540 rpm; 10 g/L, 0.2M, pH 6.7 and 360 rpm; and 10 g/L, 0.2M, pH 8.0 and 360 rpm) (Tables 1 and 2). Under an industry point of view, to which the secrecy of the process is a desirable target, the large variety of gelation conditions contributes to reach that aim.

Table 1: Encapsulated invertase activity (U/mL) in function of SG800 (5 g/L and 10 g/L), barium nitrate (0.1M and 0.2M) and pH (4.0, 6.7 and 8.0). The hydrolysis was carried out in the presence of 500 Ba^{2+}-SG800-invertase beads and 100 g/L of sucrose at 35°C, pH 4.6 and 450 rpm. The yields of encapsulated invertase (YIE), expressed as percentage, were calculated by the equation 1 (V_{sol} = 0.331 U/mL).

SG800 (g/L)	$Ba(NO_3)_2$ (M)	pH		
		4.0	6.7	8.0
5	0.1	[a]0.226/[b]0.217	0.201/0.172	0.209/0.233
	YIE (%)	68.2/65.6	60.7/52.0	63.1/**70.4**
	0.2	0.159/0.177	0.186/0.192	0.190/0.174
	YIE (%)	48.0/53.5	56.2/58.0	57.4/52.6
10	0.1	0.203/0.249	0.153/0.194	0.148/0.233
	YIE (%)	61.3/**75.2**	46.2/58.6	44.7/**70.4**
	0.2	0.217/0.190	0.235/0.210	0.242/0.208
	YIE (%)	65.6/57.4	**71.0**/63.4	**73.1**/62.8

[a] Stirring = 360 rpm; [b] Stirring = 540 rpm.

Table 2: Encapsulated invertase activity (U/mL) in function of S1100 (5 g/L and 10 g/L), barium nitrate (0.1M and 0.2M) and pH (4.0, 6.7 and 8.0). The hydrolysis was carried out in the presence of 500 Ba^{2+}-S1100-invertase beads and 100 g/L of sucrose at 35°C, pH 4.6 and 450 rpm. The yields of encapsulated invertase (YIE), expressed as percentage, were calculated by the equation 1 (V_{sol} = 0.331 U/mL).

S1100 (g/L)	$Ba(NO_3)_2$ (M)	pH		
		4.0	6.7	8.0
5	0.1	[a]0.174/[b]0.140	0.168/0.141	0.171/0.141
	YIE (%)	52.3/42.3	50.8/42.6	51.7/42.6
	0.2	0.174/0.164	0.170/0.157	0.173/0.147
	YIE (%)	52.6/49.5	51.4/47.4	52.3/44.4
10	0.1	0.185/0.166	0.151/0.166	0.143/0.213
	YIE (%)	55.9/50.2	45.6/50.2	43.2/64.4
	0.2	0.166/0.192	0.218/0.167	0.152/0.167
	YIE (%)	50.2/58.0	66.0/50.5	46.0/50.5

[a] Stirring = 360 rpm; [b] Stirring = 540 rpm.

The kinetic constants (K_M and V_{max}) were determined using the conventional Hanes-Woolf plot (Figure 3) for soluble and encapsulated Ba^{2+}-SG800 invertase. The initial invertase activity was obtained from initial sucrose concentration (S) varying from 5.5 mM to 45 mM. The minimal square linear regression equations are:

$(S/V)_{sol} = 2.72S + 144$ (r = 0.9993) (Eq. 9)

$(S/V)_{encap} = 3.38S + 25.4$ (r = 0.9997) (Eq. 10)

The kinetic constants calculated using the equations 9 and 10 for soluble and encapsulated invertase, respectively, were K_M = 52.9 mM, V_{max} = 0.368 U/mL, $(K_M)_{encap}$ = 7.51 mM and $(V_{max})_{encap}$ = 0.296 U/mL.

The K_M of 52.9 mM for soluble invertase is in accordance with that found in the literature, whose values vary between 40 and 166 mM.[12][13][14] The difference observed could be due to the different origin of enzymes and assay conditions. The $(K_M)_{encap}$ determined for Ba^{2+}-alginate-invertase (7.51 mM) was lower than that described in the literature, such as for invertase entrapped in beads of polyacrylamide-gelatine (166mM)[12] or in polyethylenimine-grafted poly(GMA-MMA) (29mM).[13] However, the $(K_M)_{encap}$ of 7.51 mM was close to that calculated for Ca^{2+}-alginate invertase (7.2 mM).[14] The apparent high efficiency of invertase entrapped either in Ba^{2+} or Ca^{2+} alginate beads – which requires a low substrate concentration to reach the V_{max} - over other carriers could depend on the high diffusion of substrate and products through the bead membrane and/or aggregation degree of invertase molecules inside

the bead (monomer, dimmer, trimmer or tetramer). The latter situation could lead to invertase molecules aggregate in tetramer form, which is more active than the isolated invertase molecule.[2]

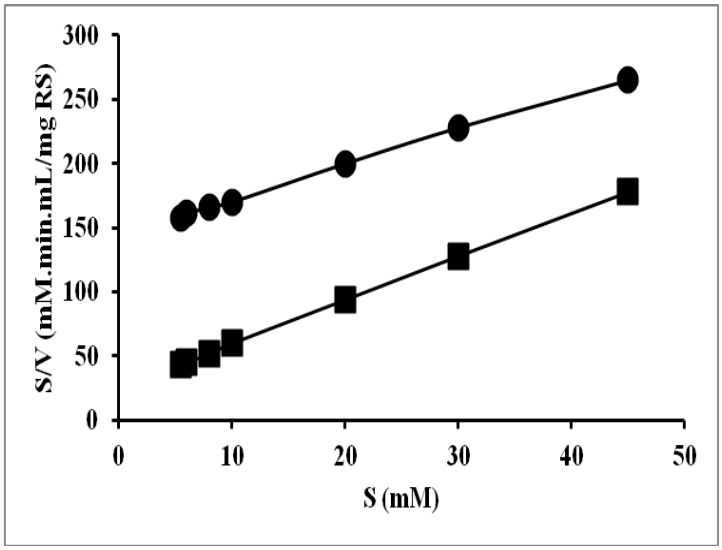

Figure 3: Hanes-Woolf plot for soluble (●) and encapsulated invertase (■). The beads were obtained by polymerizing 10 g/L of SG800 with 0.2M $Ba(NO_3)_2$ at pH 8.0, 360 rpm and 30°C.

Reusability of Ba^{2+}-SG800-invertase was analyzed by measuring its activity over three successive batch sucrose hydrolysis (Figure 4).

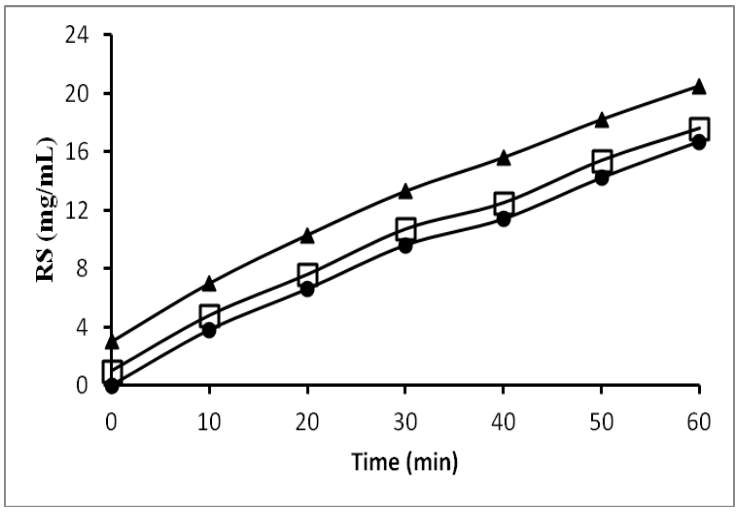

Figure 4 – Reusability of Ba^{2+}-alginate invertase beads for three successive batches [1st (●), 2nd (□) and 3rd (▲)]. The beads were obtained by polymerizing 10 g/L of SG800 with 0.1M $Ba(NO_3)_2$ at pH 4.0, 540 rpm and 30°C.

The minimal square linear regression equations are:

$Y_{1st} = 0.271X + 0.788$ (r = 0.996) (Eq. 11)

$Y_{2nd} = 0.271X + 1.82$ (r = 0.995) (Eq. 12)

$Y_{3rd} = 0.286X + 3.96$ (r = 0.994) (Eq. 13)

Where Y_{1st}, Y_{2nd} and Y_{3rd} are the RS formed (mg/mL) in the first, second and third batch, respectively; X = reaction time (min).

As can be seen in Figure 4, there was no significant difference in invertase activity. However, the initial RS concentration after the second batch is not zero, indicating an accumulation of reducing sugars inside beads, which would probably increase as batches were tested successively. Undoubtedly, the best approach for evaluating the reusability of encapsulated invertase – and by extension any kind of immobilized enzyme – would be the continuous process.[12]

CONCLUSION

The data obtained led to the conclusion that the barium consumption and the number of beads produced are linearly correlated. In addition, the invertase encapsulation yield (YIE) varied from 44.7% to 75.2% for SG800 (M/G < 1) and from 42.3% to 55.9% for S1100 (M/G > 1), whereas the encapsulated invertase activity varied for SG800 (from 0.148 U/mL to 0.249 U/mL) and for S1100 (from 0.143 U/mL to 0.218 U/mL). The YIE over 70% was obtained using SG800 alginate. The kinetic constants for soluble and encapsulated invertase were K_M = 52.9 mM, $(K_M)_{encap}$ = 7.51 mM, V_{max} = 0.368 U/mL and $(V_{max})_{encap}$ = 0.269 U/mL. Finally, the barium-alginate beads were reused three times batchwise without significant loss of invertase activity.

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