Optimization and Immobilization of alpha-amylase from *Bacillus subtilis* in calcium alginate and calcium alginate – cellulosic residue beads

Abdallah Herizi,[1,2] Rachid Souilah,[1,3] Djaafar Djabali,[1] Boubekeur Nadjemi[1]

[1]Department of Chemistry, Ecole Normale Supérieure de Kouba, Algeria; [2]Faculty of Technology, Mohamed Boudiaf M'sila University, Algeria; [3]Department of Physics, Ecole Normale Supérieure de Laghouat, Algeria

Abstract

In this study, Alpha amylase from *Bacillus subtilis* was immobilized by entrapment in Calcium Alginate beads (CA). To improve the properties of these beads, alginate was blended with Cellulosic Residue (CR) obtained from sorghum-starch extraction. The conditions of entrapment were optimized for a maximum immobilization yield (Y%) by mathematical statistics, where the 2^3-full factorial design of experiments was used. The properties of calcium alginate beads were improved by comparing the activity of immobilized enzymes in the hydrolysis of starch. The activity of the immobilized enzyme by Calcium Alginate /Cellulosic Residue (CA/CR) was found to be higher than the Calcium Alginate method. Zn^{2+} and Cu^{2+} have inhibitory effects on both immobilized enzymes. The *Bacillus subtilis* immobilized in alginate can be reused for 7 cycles with 12.7 µmol of reduced sugars and 6 cycles for the entrapped enzyme in CA/CR with 30 µmol of reduced sugars.

Introduction

α-Amylase is an important amylolytic enzyme participating in the hydrolysis of starch, the most common carbohydrate in nature compared to plant and animal origins.[1]

A-amylases have many applications in numerous industries such as food, feed, detergents, textile, pharmaceutics and paper.[2,3] Microbial α-amylase is the most popular source of industrial α-amylase. Many chemicals like ethanol, amino acids, citric acids, nitrates, nitrites, fine chemicals and essential compounds could be synthesized by biological pathways. Enzyme cat-alyzed reactions provide huge boost to cost-effective and environmentally friendly technology.[4]

Enzyme immobilization can be defined as the attachment of free or soluble enzymes to different types of supports resulting in a reduction or loss of the enzyme mobility.[5]

Among various immobilization methods, entrapment is one of the most preferable method because it prevents excessive loss of enzyme activity and protects the enzyme from microbial contamination.[6] The selection of the supporting material and the immobilization method are of great importance to obtain higher performance of the enzymatic reaction.[7] Natural polymers are usually used as structured carriers for encapsulation, especially polysaccharides group such as carrageenan,[8] chitosan[9] and starch.[10] Alginate is the most common support, for its easy formulation in, mild gelation conditions, non-toxicity, biocompatibility, low cost and resistance to microbial attacks.[11] Physical entrapment of α-amylase in calcium alginate beads has shown to be a relatively easy, rapid and safe technique.[12] Alginate beads could face problems such as distorted shapes, uneven sizes, poor mechanical strength and high porosity. These defects may influence the stability and viability of the encapsulated enzyme during storage.[13-15]

In the present study, α-amylase was immobilized in calcium alginate gel beads. To improve the properties of the beads, alginate was blended with Cellulosic Residue (CR) and the entrapment conditions such as the concentration of sodium alginate (C_{alg}), enzyme (C_{enz}) were also optimized.

Materials and Methods

A-Amylase from *Bacillus subtilis* 50U/mg (10070), and soluble starch were purchased from Sigma-Aldrish. Sodium alginate from *Laminaria digitata algae* (M/G=1.2).[16-19] CR obtained after sorghum starch extraction. All the other chemicals used were of analytical grade.

Assay of α-amylase activity

Amylolytic enzyme activity was assayed by measuring the reduced sugars released during the reaction, and its action on soluble starch from Zulkowsky (1% w/v) using dinitrosalicylic acid (DNS) reagent.[20] Where one unit will release 1µmol of maltose from starch in 3 minutes at pH 6.9 and 20°C.

Enzyme immobilization

Entrapment of the enzyme in calcium alginate and (CA/CR) capsule was carried out by extruding through a Pasteur pipette the mixture of aqueous sodium alginate or Calcium Alginate /Cellulosic Residue with α-Amylase into a gently stirred 0.1 M $CaCl_2$ solution. The formed capsules were recovered by filtration using a Buchner funnel and thoroughly washed with distilled water in order to remove excess of $CaCl_2$ and non-trapped amounts of the enzyme.[12,15,17]

Immobilization yield (Y%)

The immobilization yield (Y%) was calculated by using Eq1[21]:

$$\text{Immobilization yield}(Y\%) = \frac{\text{activity of immobilized enzyme}}{\text{activity of soluble enzyme}} \times 100 \quad (Eq1)$$

Protein determination

The amount of immobilized enzymes was determined by the Kjeldhal method.[22]

Optimization of immobilization parameters in alginate beads

Different sodium alginate concentrations (0.66-2% w/v), enzyme concentrations (0.025-1% w/v) and stirring time (30-120 min) were used during immobilization of α-amylase in alginate beads to obtain a high immobilization yield (Y%).

Optimization of immobilization parameters in CA/CR beads

Different sodium alginate concentra-

tions (0.66-2% w/v), enzyme concentrations (0.025-1% w/v) and (CR) concentrations (0.1-2%w/v) were used during immobilization of α-amylase in CA/CR to obtain a high immobilization yield (Y%).

Statistical optimization of the parameters

The purpose of statistically designing an experiment is to collect the maximum amount of relevant information with a minimum expenditure of time and resources.[23] Statistical design of experiments is a powerful tool for optimizing processes.[24] In order to maximize the immobilization yield (Y%), full factorial design for three independent variables was adopted. The variables are the alginate concentrations (C_{alg}), enzyme (C_{enz}) and the stirring time (t_s) for the first immobilization method and the alginate concentrations (C_{alg}), enzyme (C_{enz}) and CR for the second method.

For statistical calculation, the variables have been coded as xi according to Table 1 and Table 2.

The 2^3-full factorial designs and the applied mathematical model form a first order regression equation is:

$$Y= b_0 +b_1X_1+b_2X_2+b_3X_3+b_{12}X_1X_2+b_{13}X_1X_3 +b_{23}X_2X_3+b_{123}X_1X_2X_3$$

Whereas is the predicted response, b_0 the offset term, b_i the linear effect, and b_{kj} the interaction effect. This equation was optimized for maximum value to obtain the optimum conditions.[25,26] The significance of each factor was analyzed using Minitab 17 statistical software (Minitab Inc., State College, PA, USA).

Effect of metal ions on the activity of immobilized enzyme

In the enzyme action, metallic cofactors are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, all metal ions were used in chloride salts form such as Ba^{2+}, Ca^{2+}, Ni^{2+}, Sn^{2+}, Cu^{2+} and Zn^{2+}. With a Pasteur pipette the mixture of aqueous sodium alginate or sodium alginate (CR) with α-Amylase was extruding through into a gently stirred to 0.1 M salts solutions. The activity of immobilized enzyme was measured in the same way as mentioned above.

Reusability of the entrapped enzymes

To test the reusability of α-amylase entrapped were used to assay enzyme activity. After incubation, the beads were removed from the reaction mixture and reused after being washed with distilled water. The activity was determined in the same manner as described for enzyme assay. The decrease in the activity for each cycle was determined assuming a 100% activity of beads in the first cycle.

Results and Discussion

Statistical optimization of the immobilization parameters in alginate and CA/CR beads

The experimental results of the activity report recovered by immobilization of enzymes (Y%) are given in Table 3.

The results of the experimental data were studied and interpreted by Minitab 17 statistical software. The behavior of the system was explained by the following equations:

For the entrapment in Calcium alginate

$$Y = 36.860 + 7.775\,x_1 - 5.77\,x_2 + 3.10\,x_3 - 0.005 x_1\,x_2 - 7.615\,x_1\,x_3 + 2.455\,x_2\,x_3 + 7.08 x_1\,x_2\,x_3$$

For entrapment in Calcium Alginate /Cellulosic Residue (CA/CR).

$$Y = 40.055 - 12.347\,x_1 - 14.330\,x_2 + 11.790\,x_3 + 3.347 x_1\,x_2 - 12.912\,x_1\,x_3 + 1.355\,x_2\,x_3 + 8.042 x_1\,x_2\,x_3$$

The model showed that the maximum immobilization yield (Y%) in calcium alginate, is attained for an alginate concentration of 0.66% w/v, an enzyme concentration of 0.025% w/v and a stirring time of 120 min. However, the maximum immobilization yield (Y%) in CA/CR, is attained for an alginate concentrations of 0.66% w/v, an enzyme concentrations of 0.025% w/v and a residue cellulosic concentrations of 2% w/v and the maximum immobilization yield (Y%) in CA/CR was 2 fold higher than that of the maximum immobilization yield (Y%)

Table 1. Selected values of the independent variables (entrapment in Calcium Alginate).

Variables	Coded	Range -1	Range +1
C_{Alg} (%m/v)	x_1	0.66	2
C_{enz} (% m/v)	x_2	0.025	0.1
t_S (min)	x_3	30	120

C_{alg}: Alginate concentration; C_{enz}: Enzyme concentration; t_s: Stirring time.

Table 2. Selected values of the independent variables (entrapment in CA/CR).

Variables	Coded	Range -1	Range +1
C_{Alg} (%m/v)	x_1	0.66	2
C_{enz} (% m/v)	x_2	0.025	0.1
C_{CR} (% m/v)	x_3	0.1	2

C_{alg}: Alginate concentration; C_{enz}: Enzyme concentration; C_{CR}: Cellulosic residue concentration.

Table 3. Experimental design matrix and measured values of the responses.

Run	X_1	X_2	X_3	The immobilization yield (Y%) entrapment in Calcium Alginate	The immobilization yield (Y%) entrapment in CA/CR
1	-	-	-	19.51	38.69
2	+	-	-	64.46	49.21
3	-	+	-	17.23	16.71
4	+	+	-	33.84	08.45
5	-	-	+	50.19	101.47
6	+	-	+	36.36	28.17
7	-	+	+	29.41	52.74
8	+	+	+	43.88	78.03

CA/CR: Calcium Alginate/Cellulosic Residue.

in CA. Similar results were reported by Abdel-Naby (1998) that *Bacillus subtilis* entrapped in Ca-alginate showed a decrease in the immobilization yield with the increase of alginate concentration**,** and the fillers materials may be added to the formulation to increase the dry mater in the beads, Increasing mechanical resistance.[27]

Effect of metal ions on the activity of immobilized enzyme

The presence of specific metallic ions like Ba^{2+}, Ca^{2+}, Cu^{2+}, Ni^{2+}, Sn^{2+} and Zn^{2+} can inhibitor enhances the amylase activity. Figure 1 present the effect of different metal ions on immobilized enzyme activity. The maximum activity of the entrapped enzyme in alginate were obtained with Ca^{2+} followed by Ba^{2+} while Cu^{2+}, Ni^{2+}, Sn^{2+} and Zn^{2+} caused total inhibition of immobilized enzyme activity in previous reports, most amylase activities were inhibited in the present of Cu^{2+}, Ni^{2+} and Zn^{2+}.[28-30] The immobilization of enzyme in CA/CR caused an increase activity of entrapped enzyme in the presence of Ni^{2+} and Sn^{2+} and enhanced the activity in the presence of Ca^{2+} and a negligible decrease with Ba^{2+}.

Reusability of the entrapped enzymes

The most important advantage of immobilization is repeated use of enzymes. Reusability of the immobilized *Bacillus subtilis* was examined by using the same conditions repeatedly.

The relative activities are shown in Figure 2. It indicates that the catalytic activity of the immobilized enzyme in alginate was durable even after 7 repeated uses. The entrapped enzyme retained 80% of its initial activity after the first run, 50% activity after 4 runs and demonstrated 80% of its activity after 7 runs and obtain 12.7 µmol of reducing sugars (Figure 4).

Figure 3 shows the relative activity of immobilized enzyme in CA/CR. It can be seen that the entrapped enzyme in CA/CR could be used for 6 times. The entrapped enzyme demonstrated 50% activity after the first run, 60% activity after the 4 runs and 65% activity after 6 runs and obtain 30 µmol of reducing sugars (Figure 4). Overall recycling test results suggest that the two entrapped enzymes can be reused for several consecutive runs.

Conclusions

This study enabled us to make several conclusions. The stability of Calcium Alginate (CA) beads improved when it was blended with Cellulosic Residue (CR). *Bacillus subtilis* entrapped in Ca-alginate and CA/CR showed a decrease in the
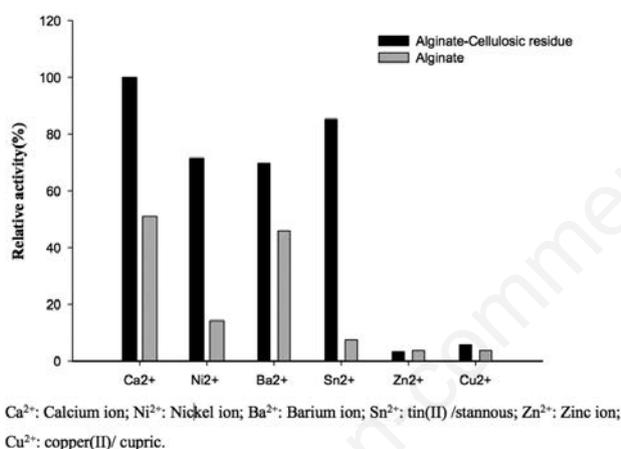

Ca^{2+}: Calcium ion; Ni^{2+}: Nickel ion; Ba^{2+}: Barium ion; Sn^{2+}: tin(II) /stannous; Zn^{2+}: Zinc ion; Cu^{2+}: copper(II)/ cupric.

Figure 1. Effect of different metal ions on immobilized enzyme activity.
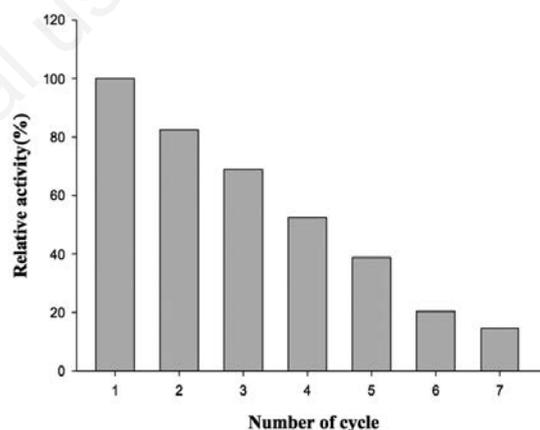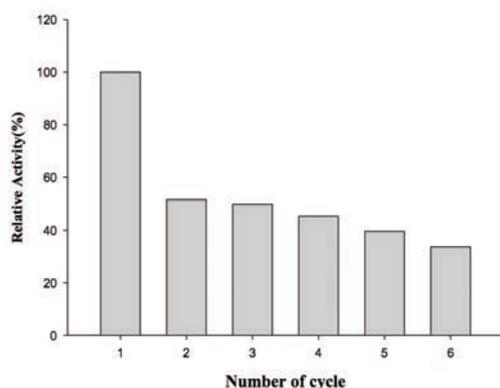

Figure 2. Reuse of entrapped enzyme in alginate.

Figure 3. Reuse of entrapped enzyme in CA/CR.

n_{sugar} (micro mol): the amount of sugar (micro mol);
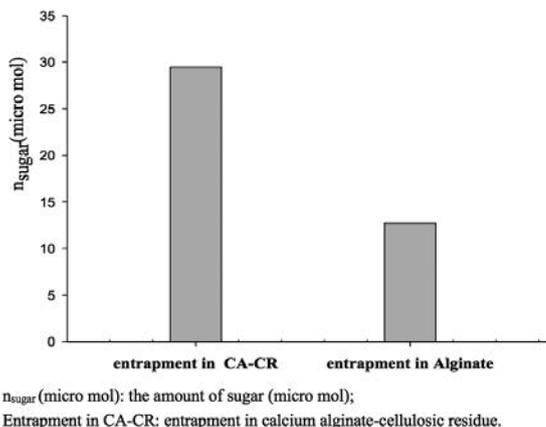Entrapment in CA-CR: entrapment in calcium alginate-cellulosic residue.

Figure 4. Total quantities of reducing sugars product in one cycle of using.

immobilization yield with the increase of alginate and enzyme concentration; *Bacillus subtilis* entrapped in Ca-alginate activity was inhibited in the present of Cu^{2+}, Ni^{2+} and Zn^{2+}. However, the immobilization in CA/CR caused an increase activity of entrapped enzyme in the presence of Ni^{2+} and Sn^{2+}.

The results suggest that the two entrapped enzymes can be reused for several consecutive runs and the quantity of reducing sugars obtained by the entrapped enzyme in CA/CR is more than 2 times greater than the amount obtained by the entrapped enzyme in alginate.

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