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High sucrolytic activity by invertase immobilized onto magnetic diatomaceous earth nanoparticles

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A B S T R A C T

Invertase immobilized on magnetic diatomaceous earth nanoparticles (mDE-APTES-invertase) with high sucrolytic activity was obtained by an easy and low-cost method. An experimental design was carried out to investigate the best immobilization conditions and it allowed obtaining an immobilized derivative with a residual specific activity equal to 92.5%. Then, a second experimental design selected the mDE-APTES-invertase with higher specific activity in relation to other derivatives reported in the literature (2.42-fold). Thermal and storage stability for immobilized invertase were found to be 35 °C for 60 min (85% retained activity) and 120 days storage period (80% retained activity), respectively. Besides, a residual activity higher than 60% and 50% were observed for mDE-APTES-invertase after reuse in short and long term, respectively. Given the simple and efficient method to obtain an immobilized derivative with high activity, the mDE nanoparticles appear to be a promising matrix for invertase immobilization as well as for other biomolecules.

1. Introduction

Immobilized enzymes have been widely used in the biotechnology. These biocatalysts have presented some advantages such as thermal stability, easy separation from reactor and reuse. These benefits lead to a reduction of process costs [1]. The industrial use of immobilized enzymes requires the evaluation of variables that may affect their activities in order to optimize their performance [2]. Invert sugar is a valuable commercial product for the food industry in countries where the main sources of sugar are beet or cane. The free and immobilized invertase (EC 3.2.1.26) produces high quality invert sugar with low concentrations of 5-hydroxymethyl-2-furfural (HMF – a carcinogenic byproduct) and without color development compared to the colored version obtained through acid hydrolysis [3,4]. Invertase is one of the most studied enzymes and it has been immobilized by different methods and supports [5–8].

Magnetic bio-separation technology is an attractive strategy for recovering magnetic immobilized enzymatic derivatives. These composites under nanoparticles sizes add the advantage of high surface to volume ratio such as the immobilized invertase on chitosan coated γ-Fe_2O_3 magnetic nanoparticles [7]. Diatomaceous earth (DE) or diatomite is a naturally occurring clay from geological deposits composed predominantly of the fossilized skeletons of diatoms. These organisms constitute an abundant and inexpensive source of silica [9]. DE typically consists of 87–94% silicon dioxide (SiO_2) with significant quantities of alumina (Al_2O_3) and ferric oxide (Fe_2O_3) [10,11]. They present interesting properties such as porous structure, high silica content, low density, high surface area, low conductivity coefficient and are chemically inert. DE has numerous applications as filter media, adsorbent, catalyst support or carrier, natural insecticide or grain protectant [10,12]. Magnetic diatomaceous earth (mDE) has been explored in magnetic bio-separation technology [13–17]. However, these applications do not include enzyme immobilization that was firstly employed for invertase in our lab [18].

In this study, mDE nanoparticles were proposed as a matrix for protein immobilization using invertase as enzyme model. In addition, our main goal was to investigate the immobilization process as well as the best conditions of the sucrose hydrolysis by the immobilized derivative through the design of experiments. Physicochemical characterization of the matrix and the immobilized invertase functionality,

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such as thermal stability, storage stability, shelf life and reuse, were also performed. All the results obtained in this work would provide a sound basis for further exploration.

2. Materials and methods

2.1. Materials

Diatomaceous earth (DE) was kindly supplied by TAMER S.A. (Salta, Argentina). A process of water washing and repeated sedimentation was applied to purify the raw DE. Invertase from Baker's yeast (178.8 U mg^{-1} protein), aminopropyltriethoxysilane (APTES), glutaraldehyde and bovine serum albumin (BSA) were purchased from Sigma Aldrich Chemicals (St. Louis, USA). All other chemicals were of high purity available commercially.

2.2. Diatomaceous earth magnetization

Magnetic DE nanoparticles (grain size around of 12 nm) were synthesized as reported by Cabrera et al. [18]. Briefly, 5 mL of iron solutions (0.6 mol L^{-1} $FeCl_2{\cdot}4H_2O$ and 1.1 mol L^{-1} $FeCl_3{\cdot}6H_2O$) were added to DE preparation (2.0% w/v). The final pH magnetization was adjusted with ammonium hydroxide (7.6 mol L^{-1}) up to 11.0. After this, magnetic solution was kept under stirring for 30 min at 100 °C. The magnetic diatomaceous earth (mDE) obtained (black precipitate) was washed with distilled water until pH 7.0 by using a magnetic field (Ciba Corning; 0.6 T). The mDE nanoparticles were stored at 25 °C until later use. This magnetic preparation is economically attractive due to the low cost of the diatomaceous earth found in natural resource as well as the co-precipitation method for magnetite nanoparticles synthesis is also very simple and fast.

2.3. Invertase immobilization on mDE-APTES:1st Experimental design

The mDE functionalization with APTES/glutaraldehyde, and posterior immobilization process were investigated using design of experiments (DOE). This statistical analysis is a structured and systematized method of experimentation in which all factors are varied simultaneously over a set of experimental runs [19]. Here, we used this method to identify the effects of several variables on the mDE functionalization and invertase immobilization process. Thus, a 2_{IV}^{7-2} fractional factorial design including seven variables with two levels namely low (−1) and high (+1) was employed. The variables under study were: APTES concentration, APTES contact time, glutaraldehyde concentration, glutaraldehyde contact time, immobilization time, immobilization pH and invertase concentration. Table 1 shows the range of the studied variables and the experimental runs. The sequence of experiments was randomized in order to minimize the effects of the uncontrolled factors.

Preparation of mDE-APTES. The mDE nanoparticles (0.10 g) were submerged in a silane coupling agent solution (2 mL APTES, prepared in acetone) and stirred at 25 °C. Next, the mDE nanoparticles treated with APTES (mDE-APTES) were washed with distilled water and recovered using magnetic field (0.6 T).

Preparation of mDE-APTES-invertase. Firstly, the activation of the mDE-APTES (0.01 g) with glutaraldehyde (2 mL) prepared in 0.2 mol L^{-1} sodium acetate buffer pH 5.0, from now on called buffer, at 25 °C was also necessary to immobilize the enzyme by covalent binding. The mDE-APTES activated with glutaraldehyde was washed several times with distilled water and buffer until the washings became colorless. After this, invertase solution (1 mL, prepared in buffer) was incubated with mDE-APTES activated (0.01 g) at 4 °C under mild stirring for different times (see Table 1). The washing procedure was repeated for five times with buffer. The invertase immobilized on mDE-APTES (mDE-APTES-invertase) was collected using external magnetic field and the supernatants including the first two washings were used for protein determination according to Lowry et al. [20] using BSA as standard. The amount of immobilized protein was calculated by the difference between the offered protein and that found in the supernatants and washings. The mDE-APTES-invertase was stored in buffer at 4 °C for further use. The immobilization pH and time were also investigated in order to study their influences in the immobilization process (see Table 1). For this, invertase solutions were prepared at different pH (0.2 mol L^{-1} sodium acetate buffer), and immobilization time was set up according to the time variation in the procedure of immobilization.

Table 1
Experimental design for the mDE functionalization processes and the covalent immobilization of enzyme.

Variables	Factor code	Unit	Variables levels	
			−1	+1
[APTES]	X_1	%	2.5	10.0
APTES contact time	X_2	h	1	2
[Glutaraldehyde]	X_3	%	2.5	10.0
Glutaraldehyde contact time	X_4	h	1	2
Immobilization time	X_5	h	2	12
Immobilization pH	X_6	–	4.0	5.5
[Invertase]	X_7	mg mL^{-1}	0.05	0.15

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	Y^a	Y^b
1	−1	−1	−1	−1	-1	+1	+1	36	0.330
2	+1	−1	−1	−1	-1	-1	-1	100	0.057
3	−1	+1	−1	−1	-1	-1	-1	100	0.093
4	+1	+1	−1	−1	-1	+1	+1	47	0.616
5	−1	−1	+1	−1	-1	-1	+1	88	0.182
6	+1	−1	+1	−1	-1	+1	-1	80	0.199
7	−1	+1	+1	−1	-1	+1	-1	83	0.278
8	+1	+1	+1	−1	-1	-1	+1	87	0.085
9	−1	−1	−1	+1	-1	-1	-1	100	0.094
10	+1	−1	−1	+1	-1	+1	+1	27	0.354
11	−1	+1	−1	+1	-1	+1	+1	34	0.324
12	+1	+1	−1	+1	-1	-1	-1	100	0.057
13	−1	−1	+1	+1	-1	+1	-1	92	0.532
14	+1	−1	+1	+1	-1	-1	+1	94	0.185
15	−1	+1	+1	+1	-1	-1	+1	98	0.183
16	+1	+1	+1	+1	-1	+1	-1	91	0.102
17	−1	−1	−1	−1	+1	+1	-1	100	0.100
18	+1	−1	−1	−1	+1	-1	+1	79	0.209
19	−1	+1	−1	−1	+1	-1	+1	79	1.043
20	+1	+1	−1	−1	+1	+1	-1	66	0.420
21	−1	−1	+1	−1	+1	-1	-1	100	0.735
22	+1	−1	+1	−1	+1	+1	+1	27	0.626
23	−1	+1	+1	−1	+1	+1	+1	36	0.886
24	+1	+1	+1	−1	+1	-1	-1	100	0.481
25	−1	−1	−1	+1	+1	-1	+1	75	0.251
26	+1	−1	−1	+1	+1	+1	-1	72	0.701
27	−1	+1	−1	+1	+1	+1	-1	73	0.765
28	+1	+1	−1	+1	+1	-1	+1	78	0.330
29	−1	−1	+1	+1	+1	+1	+1	27	0.620
30	+1	−1	+1	+1	+1	-1	-1	100	0.694
31	−1	+1	+1	+1	+1	-1	-1	100	0.566
32	+1	+1	+1	+1	+1	+1	+1	28	0.493

Y^a corresponds to the immobilized protein percentage.
Y^b corresponds to the response: enzymatic activity (U mg^{-1} support).

2.4. Sucrose hydrolysis by mDE-APTES-invertase: 2nd Experimental design

To perform the present statistical study, the experimental conditions used in the functionalization and immobilization processes were corresponding to the best run of the first experimental design. A 2^4 complete factorial design with central point was performed to investigate the best conditions of sucrose hydrolysis by mDE-APTES-invertase. This approach enabled an experimental investigation of the individual factors and the interactions of the factors simultaneously as opposed to one factor at-a-time approach. The independent variables studied were:

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