Colloid Polym Sci (2017) 295:495–506
DOI 10.1007/s00396-017-4024-x

ORIGINAL CONTRIBUTION

A novel alginate–CMC gel beads for efficient covalent inulinase immobilization

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Received: 25 August 2016 / Revised: 9 January 2017 / Accepted: 21 January 2017 / Published online: 9 February 2017

Abstract Covalent immobilization of inulinase, produced by the marine-derived fungus *Aspergillus terreus*, on novel gel beads was made by the combination of alginate and carboxymethyl cellulose. Optimization of the loading time and loading units was done by response surface methodology. The bound enzyme displayed a change in optimum operating pH from 5.0 to 5.5 while the optimum operating temperature increased from 50 to 55 °C. K_m value has been increased (from 3.6 to 7.1 mg/ml) in comparison with the free enzyme. However, the V_{max} was lowered (from 145 to 77.5 U/g carrier) after immobilization. The immobilized inulinase showed enhancement in thermal stability against high temperature. There was an observed increase in half-lives and *D* values which revealed the improvement in the enzyme thermal stability. Thermodynamically, after immobilization, a remarkable increase in enthalpy and free energy was observed due to the enhancement of enzyme stability. Immobilized inulinase showed retention of 60% of its original activity after 10 successive cycles. Stability and reusability of immobilized inulinase on alginate–CMC enable the enzyme to be more convenient for industrial application.

Keywords Inulinase · Covalent immobilization · Response surface methodology · Alginate · Carboxymethyl cellulose

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Introduction

Inulinases catalyze inulin hydrolysis to produce inulo-oligosaccharides, fructose, and glucose. These can be classified into two types: endoinulinase (E.C. 3.2.1.7) that produces inulo-oligosaccharides) IOS) and exoinulinase (E.C. 3.8.1.80) which splits the terminal units of fructose. Exoinulinase is used for the production of high-fructose syrup (HFS) from inulin, while endoinulinase can be used for production of inulo-oligosaccharides of different lengths [1]. Fructose has a higher sweetening power compared to sucrose (about 70% higher), and it is used as a sweetener in food and beverage industries. It is well tolerated by diabetics, improves iron absorption by children, and helps in the removal of ethanol from the blood of alcoholics [2]. Fructo-oligosaccharides (FOS) increase the population of the gut bifidobacteria and improve the absorption of minerals [3].

The use of immobilized enzymes in industrial processes is more advantageous than the free forms as they help in the separation of reactants and products, allow the recovery of the enzyme for reuse, increase the enzyme stability, and reduce the costs of the process. Additionally, immobilization increases the selectivity of enzymes and reduces its inhibition by the products [4–7].

Many immobilization techniques were used previously for enzyme immobilization, such as gel entrapment [8], cross-linking [9, 10], physical adsorption [11], and covalent binding. Some authors have worked for many years on enzyme covalent binding immobilization using natural hydrogels and performing different modifications on gel formation to increase the enzyme resistance at high temperature and also to improve the enzyme reusability and the shelf stability of the enzymes [12–16].

The response surface method (RSM) is a combination of mathematical and statistical techniques for experimental

designing, model planning, and studying the effects of some factors to obtain the most favorable conditions. The RSM was established as an appropriate method to develop the optimal conditions or the region that fits the operation specification. The optimum conditions for enzyme immobilization have been reported frequently [17].

In the present work the marine-derived *Aspergillus terreus* inulinase was immobilized on modified grafted alginate beads. A 2^2 factorial design was employed for the planned statistical optimization of immobilization. The catalytic properties of both the free and immobilized enzymes were studied. Moreover, the shelf stability and reusability were studied for the development of continuous use in industrial processes.

Materials and methods

All experiments were carried out in triplicate and data are means ± SD (n = 3).

Chemicals

Sodium alginate (Alg) was obtained from Fluka. Carboxymethyl cellulose (CMC) was purchased from Sigma; polyethyleneimine (PEI) (MW: 423), cat no. 468533, was obtained from Aldrich; and inulin was obtained from Sigma. Crude inulinase was prepared in our laboratory. Other chemicals were of Analar or equivalent quality. Inotech Encapsulator, model IE-50, was purchased from Inotech Encapsulator in Switzerland.

Microorganism and its maintenance media:

The marine-derived *A. terreus* was used in this study as a source for inulinase enzyme. *A. terreus* was isolated from decayed wood samples collected from Ismailia, Egypt [18]. The fungal isolate was routinely grown on malt extract agar medium at 27 °C and preserved at −80 °C in 50% (*v*/*v*) glycerol.

Cultivation conditions and crude enzyme extraction

Three grams of artichoke leaves were taken into 250-ml Erlenmeyer flasks and moistened with 15 ml of Tris–maleate buffer, 0.1 M, pH 8.5 contained (g %) 0.8, KH_2PO_4; 1, $NH_4H_2PO_4$; 1, glucose; 1, sucrose; 0.006, $CaCl_2$; and 0.5 $MgSO_4$ [18]. Flasks were autoclaved for 20 min at 121 °C and cooled to room temperature before inoculation. Sterilized solid substrate was inoculated with 1.0 ml inoculum containing (5×10^6 spores/ml) of 5 days old culture prepared by inoculating a slant of the fungus in 50 ml of malt extract broth medium. The contents of the inoculated flasks were well mixed and incubated at 28 °C for 10 days. At the end of incubation period, 50 ml of sodium acetate buffer 0.1 M, pH 4.0, was added to the cultures and placed on a rotary shaker for 30 min. The suspension was filtered through a nylon cloth, followed by centrifugation at 5000 rpm for 15 min at 4 °C. Inulinase was precipitated from the culture filtrate with 60% acetone and then used for immobilization during the study.

Determination of enzyme activity

Inulinase activity was assayed by measuring the amount of the reducing sugar fructose released from inulin by Nelson's method [19]. The reaction mixture containing 0.5 ml of enzyme extract (0.5 g immobilized enzyme) and 0.5 ml of 1% (*w*/*v*) inulin in 0.2 M sodium acetate buffer (pH 5) was incubated at 50 °C for 15 min. The amount of reducing sugars released was measured using Somogyi's copper reagent. Absorbance was read at 520 nm. One unit of inulinase (IU) was defined as the amount of enzyme which librated 1 μmol of fructose per min under the assay conditions.

Preparation and grafting of alginate beads (Alg + CMC/$Ca2^+$/PEI/GA)

For gel beads formation, solutions of sodium alginate (Alg) and carboxymethyl cellulose (CMC) in a concentration of 2 and 3% (*w*/*v*), respectively, mixed together. The Alg–CMC solution was dropped through a nozzle of 300 μm using the Inotech Encapsulator in a hardening solution containing 2% (*w*/*v*) $CaCl_2$ (Ca^{2+}). The generated beads were soaked for 3 h in 1% PEI solution. The unreacted PEI was then removed from the beads by successive washing with distilled water. After washing, aminated gel beads were soaked in glutaraldehyde (GA) solution of 2.5% (*v*/*v*) for 3 h to incorporate the new functionality, aldehyde group, and then gel beads were washed with distilled water to remove unreacted GA [17]. After that, the activated gel beads were ready for immobilization step as shown in Scheme 1 for covalent immobilization of enzyme.

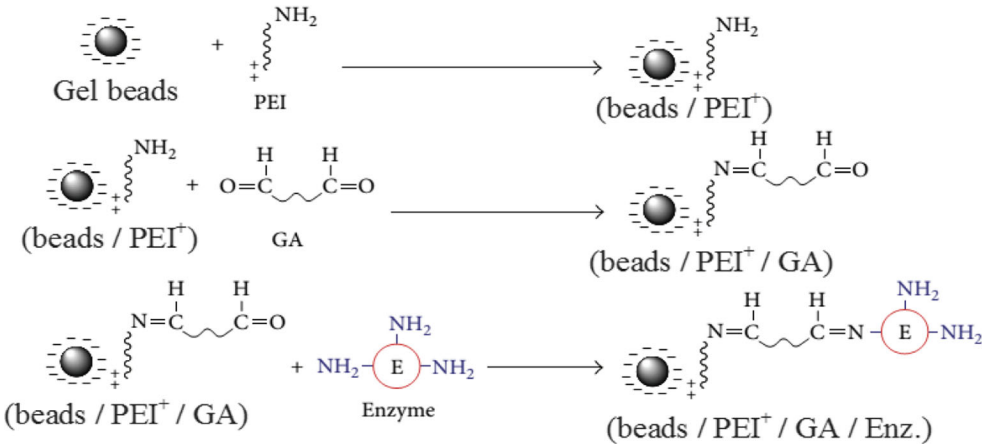
Enzyme immobilization

One milliliter of the partially purified inulinase was mixed with 1 g of the prepared grafted alginate carrier .The mixture was left for desired time at 4 °C. At the end of incubation period gel, beads were washed twice with distilled water and used for inulinase assay.

Elucidation of the modified gels using ATR–FTIR

The attenuated total reflectance-Fourier transform infrared (ATR–FTIR) has been used to identify the new functionalities on the grafted gels. IR transmission spectra were obtained using a FTIR spectrophotometer (FTIR-8300, Shimadzu,

Scheme 1 Mechanism of preparation of gel beads and enzyme immobilization



Japan). The test is aiming to prove the presence of the new functional group; carbonyl group formed at all the different formulas. A total of 2% (*w*/*w*) of the sample, with respect to the potassium bromide (KBr; S. D. Fine Chem) disk, was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before it was compressed into a KBr disk under a hydraulic press at 10,000 psi. Each KBr disk was scanned 16 times at 4 mm/s at a resolution of 2/cm over a wave number range of 400–4000/cm, using Happ–Genzel apodization. The characteristic peaks were recorded.

Differential scanning calorimetry and thermal gravimetric analysis

Thermal gravimetric analysis (TGA) was performed to prove the formation of a strong polyelectrolyte complex between the Alg–CMC and the PEI followed by Alg–CMC + PEI and GA. The thermal behavior of the different gel formulations Alg–CMC, Alg–CMC + PEI, Alg–CMC + PEI + GA, and Alg–CMC + PEI + GA + enzyme was characterized by the DSC (SDT 600, TA Instruments, USA). Approximately 3–6 mg of the dried gels was weighed into an alumina pan. TGA was performed by using (Q500 TA instrument, New Castle, DE, USA). A typical sample was heated from 50 to 300 °C at a heating rate of 10 °C/min, under a constant nitrogen flow rate of 100 ml/min.

Optimization of the enzyme loading capacity and loading time using grafted Alg–CMC gel beads using 2^2 full-factorial central composite experimental designs

Optimization of loading capacity and loading time of inulinase on the Alg–CMC gel beads was carried out by using 2^2 full-factorial central composite design [17, 20] with four-star points (±∞) and three replicates at the center point. Design matrix of 11 trials experiment (Table 1) shows the coded and actual values. The independent variables, loading time (X_1), and enzyme unit solution (X_2) were fitted with a second-order polynomial function to correlate the relationship between independent variables and response of immobilized units per gram gel disk/bead as follows:

$$Y_{\text{Activity}} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_1 \beta_2 X_1 X_2$$

where Y_{Activity} is the predicted amounts of inulinase per gram gel beads, i.e., U/g beads. β_0 is the intercept, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients, and β_{12} is cross-product coefficient. Statistical software SPSS (version 16.0) was used for the regression analysis of the experimental data obtained. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). Statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficients to determine each variable; the quadratic models were represented as contour plots (3D), and response surface curves were generated by using STATISTICA (0.6). Experiments were performed in triplicate and mean values were given.

Optimization and stability of the free and immobilized inulinases

To validate the efficiency of the novel carriers for immobilization of inulinase to be used in industries, the following experiments were conducted.

Effect of pH on immobilized and free inulinases

To determine the optimum pH for the free and immobilized inulinases, the enzymes were incubated at 50 °C for 15 min into 1 ml of 1% (*w*/*v*) inulin dissolved in 0.2 M sodium acetate buffer (pH 4.0–7.0). The data were normalized to 100% activity. The highest enzyme activity is expressed as 100%, and

Table 1 Experimental results of CCD for inulinase activity

Trial number	Time (h) X1		Loaded units X2		Inulinase activity (U/g beads)	
	Coded	Actual	Coded	Actual	Experimental	Predicted
1	−1	3	−1	5	26	22.25
2	−1	3	+1	15	30	32.104
3	+1	12	−1	5	38.5	38.283
4	+1	12	+1	15	41	43.72
5	−∞	1	0	10	23	25.45
6	+∞	24	0	10	46	45.60
7	0	6	−∞	2	19	21.903
8	0	6	+∞	20	35	32.95
9	0	6	0	10	37	35.775
10	0	6	0	10	37	35.775
11	0	6	0	10	37	35.775

each pH is expressed relatively as a percentage of the 100% activity.

Effect of temperature and thermodynamics of free and immobilized enzymes

The effect of temperature on the activity of soluble and immobilized inulinase was determined by carrying out the reaction at temperatures ranging from 40 to 65 °C for 15 min. The temperature showing maximum activity was taken as optimum for the enzyme. The residual activity (%) at each temperature was calculated by considering the enzyme activity at the optimum temperature as 100%. The activation energy (E_a) of catalysis for both free and immobilized inulinase forms was determined from the slope of the Arrhenius plot [log V (logarithm of % residual activity) versus reciprocal of absolute temperature in Kelvin (1000/T)], which is given by the following expression.

$$\text{Slope} = -\frac{E_a}{R}$$

To study the thermal stability, both free and immo bilized enzyme forms were pre-incubated at different temperatures ranging from 50 to 65 °C up to 90 min. Samples were withdrawn at intervals of time and analyzed for activity in standard enzyme assays at the optimum temperature. The residual activity was calculated by taking the enzyme activity at 0 min incubation as 100%. Results were also expressed as first-order thermal deactivation rate constants (k_d), half-lives ($t_{1/2}$), and D values (decimal reduction time or time required to pre-incubate the enzyme at a given temperature to maintain 10% residual activity) at each temperature. The k_d was determined by regression plot of log relative activity (%) versus time (min). The $t_{1/2}$ and D value of immobilized inulinase were determined from the relationships.

$$t_{1/2} = \frac{\ln 2}{k_d}$$

$$D \text{ value} = \frac{\ln 10}{k_d}$$

The temperature rise necessary to reduce D value by one logarithmic cycle (z value) was calculated from the slope of the graph between log D versus T (°C) using the equation

$$\text{Slope} = \frac{-1}{z}$$

The activation energy (E_d) for inulinase denaturation was determined by a plot of log denaturation rate constants (ln k_d) versus the reciprocal of the absolute temperature (K) using the equation

$$\text{Slope} = \frac{-E_d}{R}$$

The change in enthalpy ($\Delta H°$, kJ/mol), free energy ($\Delta G°$, kJ/mol), and entropy ($\Delta S°$, J/mol/K) for thermal denaturation of xylanase was determined using the following equation

$$\Delta H^o = E_d - RT$$

$$\Delta G^o = -RT\ln\left(\frac{\Delta k_d h}{k_B T}\right)$$

$$\Delta S^o = \Delta H^o \frac{-\Delta G^o}{T}$$

where T is the corresponding absolute temperature (K), R is the gas constant (8.314 J/mol/K), h is the Planck constant (11.04×10^{-36} J/min), and k_B is the Boltzmann constant (1.38×10^{-23} J/K).

Inulin hydrolysis

Ten units of free and immobilized enzymes were incubated at 55 °C for 1 h at pH 5.5 into the assay mixture. Samples were withdrawn at interval times from 15 min to 120 h and analyzed for inulin hydrolysis.

K_m and V_{max} of free and immobilized inulinases

The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) of free and immobilized enzymes were calculated using Lineweaver–Burk plot. Inulin was employed as a substrate (10–100 mg/ml) dissolved in sodium acetate (0.2 M, pH 5.0) at constant pH and temperature. The Lineweaver–Burk plot (double reciprocal) method was used to obtain the Michaelis–Menten kinetic models adequate for the description of the hydrolysis of inulin by the free and immobilized enzymes. Apparent K_m and V_{max} of free and immobilized inulinases were determined by plotting $1/[S]$ against $1/[V]$, respectively.

$$[S]/V_o = 1/V_{max} \times [S] + K_m/V_{max}$$

where $[S]$ is the substrate concentration (inulin), V_o is the initial enzyme velocity, V_{max} is the maximum enzyme velocity, and K_m is the Michaelis–Menten constant and is defined only in experimental terms and equals the value of $[S]$ at which V_o equals one half V_{max}.

Operational stability and shelf stability of immobilized inulinase

The reusability of inulinase covalently immobilized on grafted alginate–CMC gel beads was studied. One gram of gel beads was incubated into 1 ml of 1% (*w*/*v*) inulin at pH 5 and 50 °C for 15 min, and the substrate solution was assayed. The same gel beads were then washed with distilled water and reincubated with another substrate solution; this procedure was repeated 13 times, and the initial activity was considered as 100%. The relative activity was expressed as a percentage of the starting operational activity.

The shelf stability was studied for the free and the immobilized enzyme over a period of 12 weeks at 4 °C. Ten grams of the immobilized enzyme containing 100 U and their equivalent of the free enzyme (100 U/ml) were stored in 0.2 M sodium acetate buffer (pH 5.0) at 4 °C. The samples were covered to avoid dehydration and loss of the buffer. A sample of the free enzyme (1 ml) or the immobilized enzyme (1 g gel beads) has been withdrawn every week and assayed for enzyme activity. The starting operational activity was considered as 100% relative activity, and data were normalized to 100% activity.

Results and Discussion:

Elucidation structure of the grafted alginate

The FTIR spectroscopic analysis of (1) the generated (Alg–CMC) gel beads, (2) the activated beads, and (3) the immobilized beads were carried out in a range varied between 400 and 4000/cm (Fig. 1). The IR spectrum of Alg–CMC beads showed characteristic beaks (curve A). A new beak at 3429/cm was displayed which was corresponding to NH_2 group, indicating the presence of the amino group on the beads surface (curve B); however, the activated beads showed two new beaks. The first one was at 1717/cm referred to the (C=O) group of glutaraldehyde-free aldehyde end, and the other beak is at 1660/cm referring to ($C{=}N^{-}$) group which resulted from the reaction of NH_2 end groups with glutaraldehyde (curve C). Moreover, immobilized beads give broader beak at 3457/cm, pointed to increasing NH_2 group's concentration. From those data, it was revealed that the processes of amination, activation, and immobilization were successful. These results were in agreement with the results of obtained by other published results [21].

TGA

The TGA thermogram of Alg–CMC, Alg–CMC/PEI, Alg–CMC/PEI/GA, and Alg–CMC/PEI/GA/enzyme gels are shown in Fig. 2, and data were indicated in (Table 2). A gradual improvement in TGA was observed with the different treatment steps of Alg–CMC with PEI and GA. The TGA of Alg–CMC was 168 °C while Alg–CMC/PEI was 198 °C, and TGA for Alg–CMC/PEI/GA was 220 °C. A remarkable increase in TGA (317 °C) was determined after enzyme immobilization [22]. It was referred that the thermal improvement of the gel might be due to the polyelectrolyte interaction formation between the $-NH^{+3}$ of the PE and the –COO– of Alg–CMC. Moreover, the hardening of the beads by using GA showed an increase in the TGA of Alg–CMC/PEI/GA up to 230 °C.

The results in (Table 2) for TGA 50% at which 50% of the polymer (alginate–CMC) has been degraded showed that the improvements in the TG might be due to the formation of a stronger crosslink of the beads due to of Schiff's base formation between the free PE's amino groups and GA. Danial et al. [13] reported that when calcium alginate was treated with PEI and followed by GA, an obvious improvement in their TGA 50% was observed.

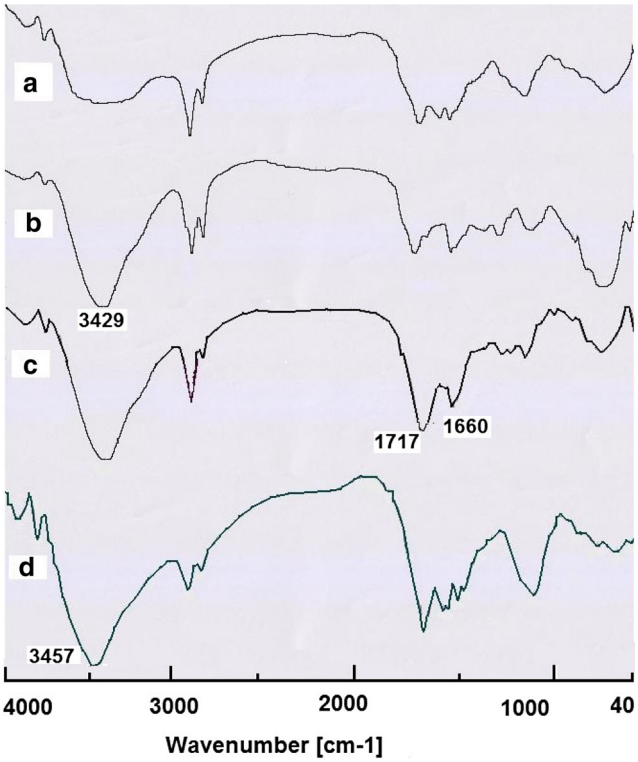


Fig. 1 FTIR of Alg–CMC beads hardened $FeCl_3$ (*A*). Alg–CMC beads treated with polyethylenimine (*B*). Alg–CMC beads treated with polyethylenimine followed by glutaraldehyde (*C*). Treated Alg–CMC beads with the inulinase (*D*)

Optimization of loading time and loading capacity by grafted Alg–CMC gel beads using 2^2 full-factorial central composite experimental designs:

The design matrix of 11 trial experiments of 2^2 full-factorial central composite design and the results are shown in Table 1. Maximum activity 46 U/g was obtained in the trial no. 6 when 10 U of inulinase incubated with one gram of Alg–CMC gel beads for 24 h. This result was very similar to the predicted value obtained by the polynomial equation (45.60 U/g beads).

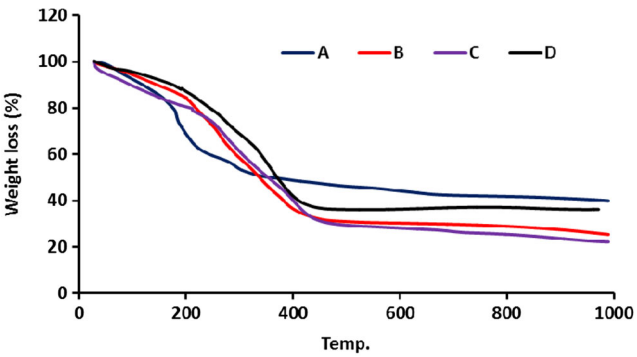


Fig. 2 TGA thermogram of five formulations covering alginate gel beads *A* (Alg–CMC), *B* (Alg–CMC + PE), *C* (Alg–CMC + PE + GA), and *D* (Alg–CMC + PE + GA + enzyme)

Table 2 TGA and $T_{50\%}$ data showing the steps of beads formation and its immobilization

Type	TGA	$T_{50\%}$
Alg–CMC	168	333
Alg–CMC + PE	198	337
Alg–CMC + PE + GA	220	353
Alg–CMC + PE + GA + enzyme	317	370

The results obtained by 2^2 full-factorial central composite design were analyzed by the standard analysis of variance (ANOVA) (Table 3). The second-order regression equation indicating the enzyme activity as a function of loading time (X_1) and amount of loading units (X_2) can be predicted by the following equation

$$Y_{\mathrm{Activity}} = 0.475 + 3.016X_1 + 3.372X_2 - 0.066X_{12} - 0.112X_{22} - 0.04X_1X_2$$

Table 3 shows the ANOVA results of inulinase immobilized onto gel beads where the amounts of enzymes loaded onto the gel beads (Y_{Activity}) were calculated the results of ANOVA and the polynomial equation. The F value was 12.52 which made the model significant. Model terms having values of Prop > F (0.007) less than 0.05 were considered significant. ANOVA indicated that the R^2 value of 0.926 for response Y_{Activity} and which indicated that 92.6% of data variability can be illustrated by the model. The observed R^2 was in agreement with the adjusted R^2 of 0.852 which assured a satisfactory adjustment of the quadratic model to the experimental data [23]. The correlation between observed and predicted results was 96.2%. All the above proved the applicability and accuracy of the central composite design for optimization of inulinase immobilization process. To explore the interaction between variables and determining the optimum value of 2^2 full-factorial central composite design, the three-dimensional response surfaces were plotted (Fig. 3). This was used by many authors for optimization of enzyme loading [17, 20].

Table 3 Analysis of variance (ANOVA) test for central composite experimental design

Terms	Response $Y_{\mathrm{inulinase}}$ activity (U/g beads)
F value	12.520
$P > F$	0.007
R	0.962
R^2	0.926
Adjusted R^2	0.852
Sundered error of the estimate	3.13737

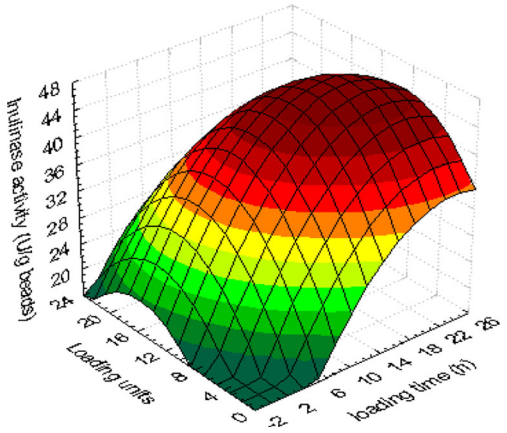


Fig. 3 Effect of the loading time and amount of loading enzyme solution (units) on inulinase activity (U/g beads)

Effect of reaction pH on free and immobilized enzyme activities

The activity of free and immobilized inulinases was tested at different pH values. The results showed in Fig. 4 showed that optimum pH values were 5 and 5.5 for free and immobilized inulinases, respectively. Immobilized inulinase had an activity at pH 5.5 which is slightly shifted in comparison with the free enzyme. Shifting to higher pH values is usually observed after inulinase immobilization [12, 13]. On the other hand, Ettalibi and Baratti, De Paula et al., and Risso et al. [24–26] observed that there is no change in the optimum pH after immobilization.

Effect of reaction temperature on free and immobilized enzyme activities

By investigating the effect of temperature, immobilized and free inulinases were assayed at different temperatures, the pH kept at the optimum. The results showed that the optimum temperature recorded for free and immobilized inulinases

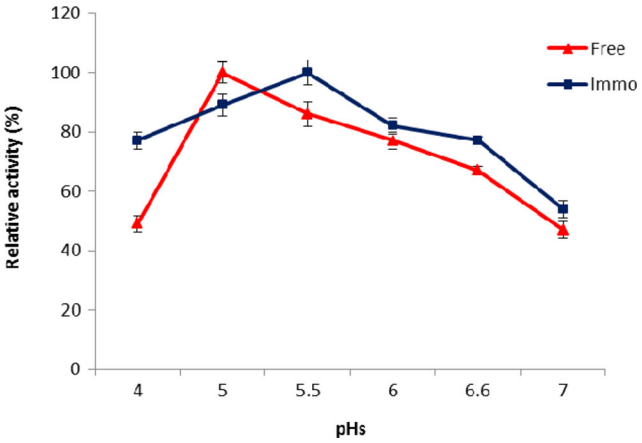


Fig. 4 pH profile of free and immobilized inulinases

were 50 and 55 °C, respectively (Fig. 5a). The formation of covalent bonds between the enzyme molecules and the carrier gave rigidity to the enzyme molecule structure, so the enzyme was less affected by temperature denaturation [27, 28]. Covalent immobilization is a technique used to engineer the enzymes to use them at high temperatures [13, 29]. High temperature was considered more interesting and preferable for improvement of conversion rates; moreover, high temperature helped in decreasing microbial contamination and allowed inulinase solubility as substrate. Conversion improvements maximize for production of the HFS by inulinase [30]. Danial et al. [13] and Sankalia et al. [31] also reported an increase in the optimum temperature for immobilized inulinase. The increase in the energy barrier of the reaction resulted from the activation energy (E_a) and Gibbs free energy ($\Delta G°$) which were calculated from Arrhenius and Eyring–Polanyi equations, respectively [32].

By using Arrhenius plot, the activation energy (E_a) of the free and immobilized inulinases was calculated (Fig. 5b). Arrhenius plot regression equations were as follows: $y = -3.4176x + 12.596$ and $y = -1.9505x + 8.0546$, respectively. The covalent immobilization of inulinase lowered the E_a from 28.41 to 16.216 kJ/mol, consequently a higher catalytic efficiency of the immobilized inulinase.

Thermal stability of the immobilized and free inulinases was also studied. The results in Fig. 6a showed that the free enzyme lost about 30% of its activity after 15 min at 50 °C

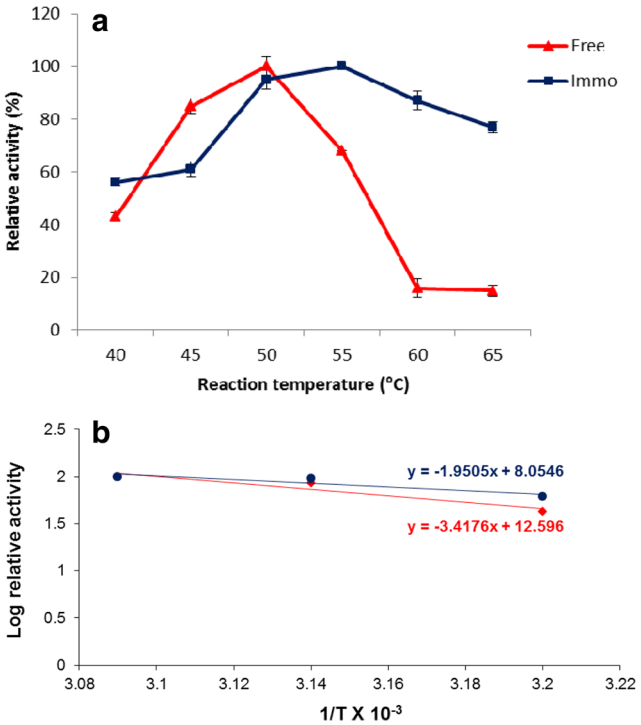


Fig. 5 **a** Optimum temperature profile of free and immobilized inulinases. **b** Arrhenius plots to calculate activation energy (E_a) for the free and immobilized enzymes

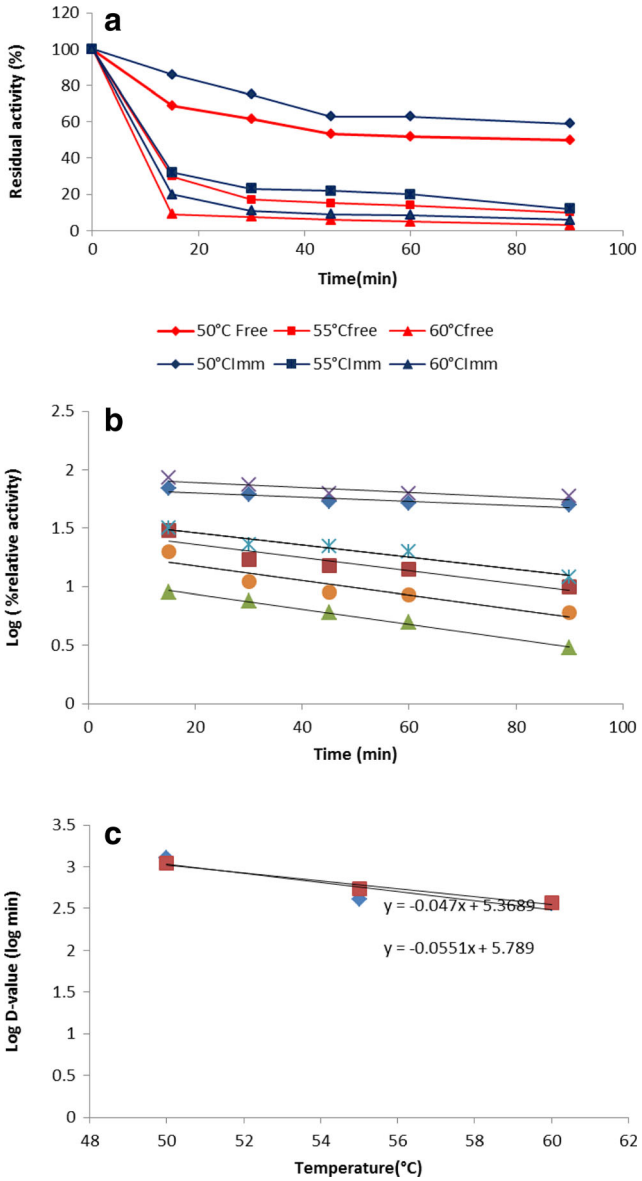


Fig. 6 **a** Temperature stability profile of free and immobilized inulinases. **b** Arrhenius plot to calculate k_d. **c** Temperature dependence of the decimal reduction of free and immobilized inulinases to calculate z values

while the results showed that immobilized enzyme retained 86% of its original activity at the same conditions. Free enzyme activity decreased to half after 60 min while the immobilized enzyme retained 63% of its activity at the same temperature. The activity of free enzyme dropped to 9% after 15 min at 60 °C while the immobilized enzyme retained about 20% of its original activity. Increasing in enzyme thermal stability and optimum temperature after immobilization indicates that the immobilization process strengthened the enzyme structure [33]. The increase in thermal stability of many immobilized enzymes is in agreement with other authors [34, 35]. Generally, the immobilization of an enzyme protects it against heat inactivation [36, 37]. Retaining of enzyme activity at higher temperatures might be due to the restriction of interaction between the immobilized enzymes molecules [38]. The thermal stability of inulinase at high temperature increases its suitability for use in industrial applications [39]. The heat inactivation rate for the immobilized enzyme was investigated at temperatures 50, 55, and 60 °C, respectively.

The plots of log of residual activity versus time were linear and indicated a first-order kinetic reaction of the immobilized enzyme (Fig. 6b). The thermostability parameters of the free and immobilized inulinases are illustrated in Table 4. The half-lives and D values of inulinase after covalent immobilization indicated better thermal stability of the immobilized inulinase. Tyagi and Gupta [34] also reported an increase in the half-life after immobilization of *Aspergillus* xylanase at 60 °C. The activation energy for thermal denaturation (E_d) was determined from the Arrhenius plot (Fig. 6c).

The E_d for immobilized inulinase was 113.7 kJ/mol which was higher than that of the free enzyme (96.65025 kJ/mol) which indicated that the immobilized enzyme was more compact, stable, and resistant to denaturation by heat. The higher value of E_d meant that we require more energy for denaturation of the immobilized enzyme [40]. A similar increase in the thermal stability after immobilization of many enzymes was observed by many researchers [34, 35].

At 50 °C, the $\Delta H°$ of free inulinase was 70.926 kJ/mol and that of the immobilized form was 87.976 kJ/mol which mean that more energy was required for the heat denaturation of the immobilized inulinase. The values of $\Delta H°$ were decreased when the temperature was increased in both cases. This indicates that at high temperatures, low energy was required for

Table 4 Kinetic and thermodynamic parameters for thermal inactivation of free and immobilized inulinases

		Free inulinase			Immobilized inulinase		
		50 °C	55 °C	60 °C	50 °C	55 °C	60 °C
Deactivation rate constants	k_d	0.0021	0.0042	0.0062	0.0018	0.0056	0.0064
Half-lives time	$T_{1/2}$	330.0701	165.035	111.7979	385.0818	123.7763	108.3042
Decimal reduction time	D value	1096.469	548.2345	371.3847	1279.214	411.1759	359.7789
The change in enthalpy	$\Delta H°$	70.92673	71.31749	71.69994	87.97648	88.36724	88.74969
Free energy	$\Delta G°$	−895.016	−862.005	−837.435	−881.932	−837.668	−819.594
Entropy	$\Delta S°$	−34.7937	−34.0273	−33.5641	−34.285	−33.0666	−32.849

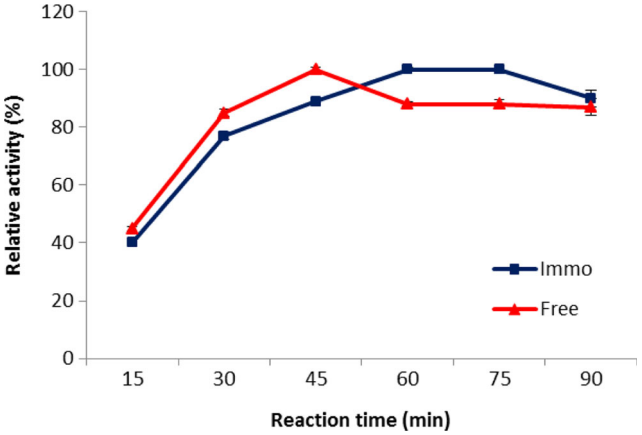


Fig. 7 Hydrolysis of inulin using the free and the immobilized inulinases

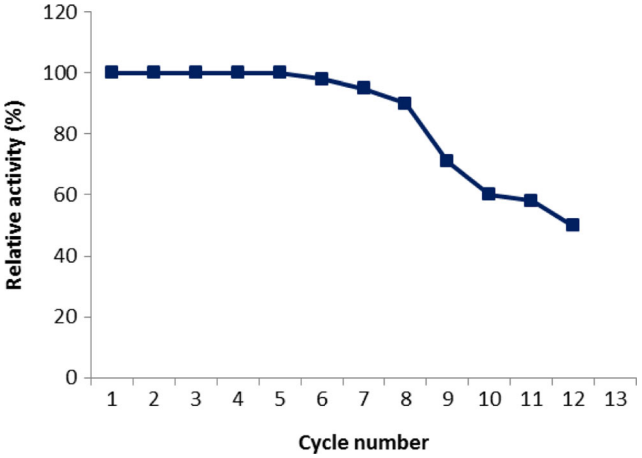


Fig. 9 Operational stability of immobilized inulinases

free enzyme denaturation but more in the case of the immobilized inulinase (Table 4). The change in $\Delta H°$ also indicated that at higher temperatures, the enzyme exhibited a conformational change in both states [41].

Gibbs free energy ($\Delta G°$) of thermal unfolding was decreased with increasing temperature and revealed a slight difference between the free and immobilized enzymes. This means that immobilization of inulinase did not affect its thermal unfolding at high temperatures. The unfolding of enzyme structure was accompanied by increasing the disorder or entropy of deactivation. However, inulinase had negative entropy ($\Delta S°$) which revealed that the native enzyme was in more ordered state (Table 4). The immobilized inulinase also had a negative $\Delta S°$ value but with a lower magnitude than in the case of the free form. The less negative value for $\Delta S°$ in the immobilized enzyme is normal due to binding with the substrate. The variation in entropy at different temperatures might be due to the changes in conformations and thermal agitation effect.

The z values of free and immobilized inulinases were calculated from the slope of the graph between log D and temperature as 18.14 and 21.27 °C, respectively. Tayefi-Nasrabadi and Asadpour [40] reported that the high z values revealed more sensitivity to heat treatment duration, and the lower z values mean more sensitivity to temperature increase. Therefore, the lesser z value of immobilized inulinase compared with the free form indicated that the immobilization

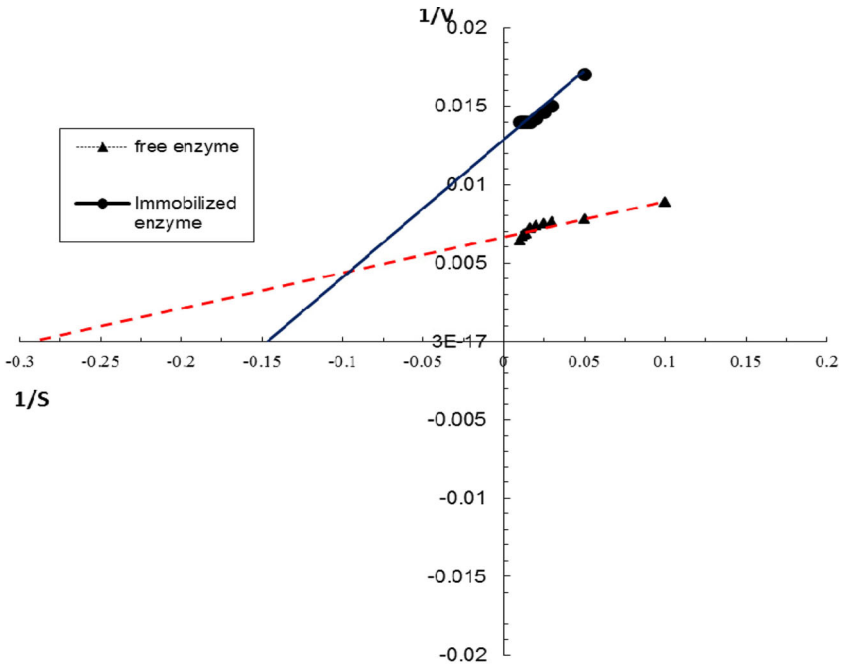


Fig. 8 Lineweaver–Burk plot for estimation of kinetic parameters for free and immobilized inulinases

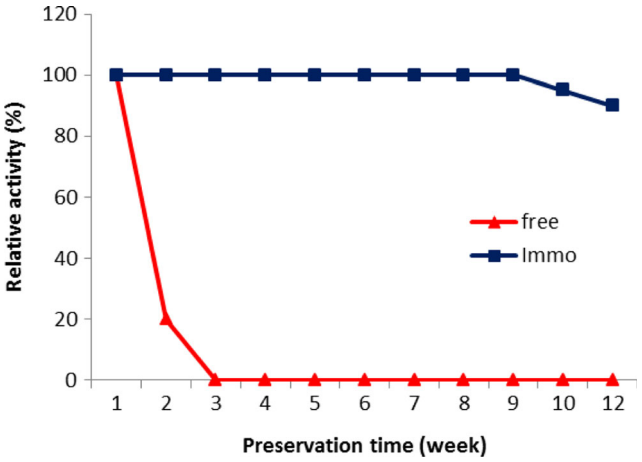


Fig. 10 Shelf stability of the free and immobilized inulinases

process made the enzyme more sensitive to temperature increase rather than the duration of heat treatment.

Hydrolysis of inulin using the immobilized and free inulinases

The results for inulin hydrolysis by immobilized and free inulinases are illustrated in Fig. 7. At 45 min, the free inulinase reached a maximum conversion rate (100%) of inulin, while the immobilized inulinase reached 100% after 60 min and onward. High concentration of product formation by immobilized enzyme represents the stability as well as a positive conformational change of the enzyme within the gel. At the same time, such increment in the reaction time also supports the possibility of resistance toward accessibility of high-molecular weight substrate to diffuse into the matrix easily [42–44].

Effect of substrate concentration on the activity of free and immobilized inulinases

The kinetic parameters regarding the substrate concentration effect for both the free and immobilized inulinases were calculated using Lineweaver–Burk plot (Fig. 8). The K_m values for free and immobilized inulinases were 3.6 and 7.1 mg/ml, respectively. An increase in K_m after enzyme immobilization might be due to the low diffusion rate of the substrate to the enzyme active site [44]. The immobilized enzymes have high K_m values especially with high-molecular weight substrates as inulin [37, 45]. The increase in K_m value after immobilization has been reported earlier by many other investigators [46, 47]. The free and immobilized enzymes had V_{max} values of 145 and 77.5 U/g, respectively. V_{max} value of the immobilized inulinase was less than that of the free enzyme and this was reported by some other authors [25, 42, 48]. A variation in the kinetic constants values could be observed due to the types of the polymer used, the variation in assay method, and the type of the inulin used. As inulin is available from different sources and has high-molecular weight; therefore, it has low dispersion ability and may cause the reduction in the efficiency of the process [42].

Operational stability of immobilized inulinase

Immobilized enzymes are preferred because they can be easily separated and reused to reduce the production costs. The inulinase immobilized on alginate–CMC beads could be reused for 10 successive cycles with retaining 60% of its original activity (Fig. 9). The enzyme retained 100% of its activity for the first five cycles after that the enzyme loss only 10% reaching the 8 cycles. The loss in activity after that was due to inactivation of the enzyme due to continuous use [17].

Shelf stability of the free and immobilized inulinases

The enzyme stability is of great significance in the production of biocatalysts. Not only the easy separation of the enzyme from its products and enzyme reusability is the main advantage of enzyme immobilization but also the immobilized enzyme stabilization during storage considered as one of the most important advantages for enzyme immobilization. The data in Fig. 10 indicated that the inulinase immobilized on grafted alginate–CMC beads retained over 100% of its activity after 9 weeks at 4 °C, while the free enzyme completely lost its activity. This may be due to the formation enzyme-gel polyelectrolyte complexes with ionic interaction which increased the shelf stability of the immobilized inulinase [17].

Conclusion

This study justified the use of a grafted alginate–CMC as a suitable matrix for the covalent binding immobilization of inulinase. The kinetic parameters of the immobilized inulinase were evaluated with reference to free inulinase. Covalent immobilization increased inulinase optimum temperature and the reaction time. This matrix also increased the inulinase stability against higher temperatures compared to the free enzyme. Immobilized inulinase beads can be reused for many consecutive cycles retaining its activity.

Compliance with ethical standards

Funding This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest The authors declare that they have no conflict of interest.

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