



# Catalytic, kinetic and thermal properties of free and immobilized *Bacillus subtilis* -MK1 $\alpha$ -amylase on Chitosan-magnetic nanoparticles

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## ARTICLE INFO

### Article history:

Received 15 May 2019

Received in revised form 31 October 2019

Accepted 26 February 2020

### Keywords:

$\alpha$ -amylase

Immobilization

Chitosan-magnetic nanoparticles

Catalytic

Kinetics

Thermodynamics

## ABSTRACT

*Bacillus subtilis* strain-MK1  $\alpha$ -amylase was successfully immobilized on Chitosan-magnetic nanoparticles (Ch-MNP) that had been modified with polyethyleneimine (PEI) and glutaraldehyde (GA). Optimization of Ch-MNP/PEI/GA beads modification by Central Composite design enhanced the immobilization yield (IY%) by 1.5-fold. Ch-MNP/PEI/GA was characterized before and after modification and immobilization by FTIR and SEM. Ch-MNP/PEI/GA/Enzyme showed the same pH optima of free enzyme, while an elevation 10 °C in temperature optima was observed after its immobilization. Ch-MNP/PEI/GA/Enzyme displayed higher  $K_m$  and  $V_{max}$  values (2.1 and 1.2-fold) and lower  $V_{max}/K_m$  ratio (1.7-fold), respectively than the free enzyme. Compared to the free enzyme, Ch-MNP/PEI/GA/Enzyme exhibited lower activation energy, lower deactivation constant rate, higher  $D$ -values, higher half-life, and higher energy for denaturation. Immobilization of  $\alpha$ -amylase increased enthalpy (4.2-fold), free energy (1.1-fold) and decreased entropy (4.6-fold) of thermal inactivation. A significant increase in pH stability of Ch-MNP/PEI/GA/Enzyme was observed especially at alkaline pH values. In addition, Ch-MNP/PEI/GA/Enzyme preserved 83.2 % of its initial activity after 15 consecutive cycles. When storing Ch-MNP/PEI/GA/Enzyme at 4 °C the residual activity was 100 and 86 %, respectively after 21 and 40 days. Finally, immobilization process improved the catalytic properties and stabilities, thus raising the suitability for industrial processes with lower cost and time.

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## 1. Introduction

Enzymes as biocatalysts are slowly but steadily gaining importance in many industries ranging from food to pharmaceuticals. The major advantage of the enzymatic route is the low toxicity, selectivity, acceleration of mild reaction conditions with its associated high yield and exclusivity towards the desired product [1].

$\alpha$ -amylase catalyzes the cleavage of  $\alpha$ -D-1,4 glucosidic bonds of starch molecule to give dextrin and other smaller polymers composed of glucose units.  $\alpha$ -amylases have diverse applications in several industries as textile, food, baking, detergent, brewing, pharmaceutical and clinical chemistry [2,3]. In spite of the important role of amylases, they exposed to some troubles as their high sensitivity to hard conditions of industrial processes, low

stability, short lifetime and difficulty in recovery which increases the production cost. To solve these problems, enzyme immobilization is considered as one of the effective techniques which not only stabilizes enzymes under operational conditions but also allows easy recovery and reusability for repeated cycles [4].

There are several methods for enzymes immobilization including covalent binding, physical adsorption, entrapment and cross linking to solid carriers. Covalent binding is considered the most effective method. The formation of covalent bonds between enzyme and carrier surface might be encouraging procedure in establishing enzymes, inhibiting their leaching out and consecutive leakage [5]. Physical adsorption is the most general, simple to perform and the oldest. The immobilized enzyme by physical adsorption shows low stability after repeated use due to the weak bonds between enzyme and carrier surface (as van der Waals, hydrogen bonding, hydrophilic/ hydrophobic and electrostatic interactions). Therefore, a method combining the advantages of physical adsorption and covalent binding was developed to enhance the immobilized enzyme stability [6].

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Immobilization can involve various carriers organic and inorganic whose characteristics play an important role in the behavior of the enzyme. Nano-carrier has a large surface area, helps to decrease the diffusion barriers in the transport of the substrate and the reaction products, thereby improving the efficiency of the immobilized enzyme [7].

The magnetite ( $\text{Fe}_3\text{O}_4$ ) nanoparticles have been considered suitable for immobilization due to their unique characteristics as their tailored surface chemistry, small size, super-paramagnetism, low toxicity, biocompatibility, biodegradability and easy separation from the reaction mixture by using external magnetic field [1,4].

For the covalent immobilization of enzymes, magnetic  $\text{Fe}_3\text{O}_4$  nanoparticles need to be coated with a few layers of chemically active natural or synthetic polymers to provide functional groups for conjugation with bio-molecule [6,7].

Chitosan is a biomaterial cheap, stable and has good hydrophilic and biocompatible properties. The amino groups of chitosan are useful for conjugation with enzyme protein via cross-linking agents such as glutaraldehyde [6].

Thermodynamic stability includes the resistance to thermal denaturation of a folded protein conformation. The suitability of enzymes for industrial application is judged by their thermodynamic parameters. Variation in thermodynamic parameters as enthalpy, entropy and the Gibbs free energy can provide valuable information about the enzyme behavior, activity and thermostability. In addition, the investigation of dynamic and thermodynamic properties of bio-nanocatalyst is important in designing a highly effective immobilized enzyme [8].

In the current study, *B. subtilis* strain-MK1  $\alpha$ -amylase was immobilized onto different carriers using covalent binding in order to increase its activity and stability. Modification of Chitosan-MNP/PEI/GA beads was optimized by using Central composite design to promote IY%. Finally, comparative studies including catalytic, kinetics and thermodynamics of both free and immobilized enzyme were carried out.

## 2. Materials and methods

### 2.1. Materials

Sodium alginate (Alg) was obtained from Fluka Company, Switzerland. Polyethyleneimine (PEI) and Glutaraldehyde (GA) were obtained from Sigma-Aldrich Chemie GmbH, Riedstr. 2, D-89555 Steinheim, Germany. Chitosan and  $\kappa$ -carrageenan were supplied from Sigma Chemical, Co., St. Louis, USA. Other chemicals were of analytical grade.

### 2.2. Production and Partial purification of $\alpha$ -amylase enzyme

$\alpha$ -amylase enzyme was produced under submerged fermentation from isolated strain *B. subtilis* strain-MK1. Cultivation of *B. subtilis* strain- MK1 cells (24 h old culture) was in 250-ml Erlenmeyer flasks containing 50 ml of sterile medium composed of (g/l): soluble starch 2.0, lactose 10.0, beef extract 5.0,  $\text{K}_2\text{HPO}_4$  10.0,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  5.0,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.25,  $\text{CaCl}_2$  0.5, pH 7.0 and incubated at 35 °C for 72 h under shaking condition of 150 rpm. The broth media after incubation was centrifuged at 10,000×g for 15 min and the cell free filtrate was considered as source of crude enzyme. The enzyme was partially purified by using ethanol precipitation method. Each precipitate obtained from 20 to 80 % concentration (v/v) was collected by centrifugation, dried, weighed and used for  $\alpha$ -amylase activity and protein estimation.

### 2.3. Enzyme assay

$\alpha$ -amylase activity was done according to [9]. For starting the reaction, 0.5 ml of 1% soluble starch in 0.1 M phosphate buffer (pH 7.0) was added to 0.5 ml of enzyme and was incubated for 30 min at 40 °C. The reaction was stopped by adding 1 ml of dinitrosalicylic acid (DNS) reagent and kept on boiling water bath for 10 min [10]. Absorbance was measured at 540 nm against blank, which was devoid of enzyme. One unit of enzyme activity (U) is defined as the amount of enzyme that liberated one  $\mu$ mole of reducing sugar as glucose/ min under assay conditions. All the experiments were performed in triplicate and the results were expressed as mean values.

### 2.4. Determination of protein content

Protein estimation was done by the method of [11] using bovine serum albumin (BSA) as standard.

### 2.5. Carriers preparation for covalent immobilization

#### 2.5.1. Alginate/ $\text{CaCl}_2$ (Alg/ $\text{CaCl}_2$ )

Sodium alginate (Alg) was dissolved in distilled water using an overhead mechanical stirrer until complete dissolution had occurred to give a final concentration of 2 % (w/v). The alginate solution was dropped through a nozzle of 300  $\mu$ m using the Innotech Encapsulator in a hardening solution containing 2.5 % (w/v)  $\text{CaCl}_2$  for 3 h [12].

#### 2.5.2. Alginate-CMC/ $\text{FeCl}_3$ (Alg-CMC/ $\text{FeCl}_3$ )

The preparation of this carrier was carried out according to [13] with some modifications. Carboxy methyl cellulose (CMC) and Alg in the ratio 3:2 were dissolved in distilled  $\text{H}_2\text{O}$  using an overhead mechanical stirrer. After complete dissolution, polymer solution was dropped into 0.05 M of ferric chloride ( $\text{FeCl}_3$ ) by using Encapsulator to form uniform gel beads. Gel beads were hardened using 0.05 M  $\text{FeCl}_3$  for 3 h.

#### 2.5.3. Alginate-CMC/ $\text{CaCl}_2$ (Alg-CMC/ $\text{CaCl}_2$ )

For gel beads formation, solutions of Alg (2%) and CMC (3%) were mixed together using an overhead mechanical stirrer until complete dissolution had occurred. The Alg-CMC solution was dropped through a nozzle of 300  $\mu$ m using the Innotech Encapsulator in a hardening solution containing 2 % (w/v)  $\text{CaCl}_2$  [14].

#### 2.5.4. Alginate-CMC/ $\text{FeCl}_3$ , $\text{CaCl}_2$ (Alg-CMC/ $\text{FeCl}_3$ , $\text{CaCl}_2$ )

For gel beads formation, solutions of Alg and CMC in a concentration of 2 and 3% (w/v), respectively, mixed together using an overhead mechanical stirrer until complete dissolution had occurred. The Alg-CMC solution was dropped through a nozzle of 300  $\mu$ m using the Innotech Encapsulator in a hardening solution containing 2% (w/v) of  $\text{CaCl}_2$  and  $\text{FeCl}_3$  (1:1 ratio) [13,14].

#### 2.5.5. CMC/ $\text{FeCl}_3$

A solution of 3 % CMC dissolved in distilled  $\text{H}_2\text{O}$  using an overhead mechanical stirrer until complete dissolution had occurred. The polymer solution was dropped through a nozzle of 300  $\mu$ m using the Innotech Encapsulator in a hardening solution 0.05 M  $\text{FeCl}_3$  [13].

#### 2.5.6. Carrageenan/KCl (Car/KCl)

Polymer carrier ( $\kappa$ -carrageenan) 2 g was dissolved in 100 ml distilled  $\text{H}_2\text{O}$ . Then, spray the polymer solution into cross-linking solution of KCl 2% (w/v), through a nozzle of 300  $\mu$ m using the Innotech Encapsulator. The prepared beads were hardened in crosslinking solutions for 3 h [15].

### 2.5.7. Chitosan/NaOH (Ch/NaOH)

Chitosan (polymer carrier) was dissolved in distilled H<sub>2</sub>O to produce 2% (w/v) solution. Then, spray the polymer solution into 5% (w/v) cross-linking solution of NaOH through a nozzle of 300 μm using the Inotech Encapsulator. The prepared beads were hardened in crosslinking solutions for 3 h [15].

### 2.5.8. Alginate-Carrageenan/KCl (Alg-Car/KCl)

Sodium alginate/*k*-Carrageenan gel (Alg-Car) was prepared by dissolving Alg/Car (1:1 w: w) in distilled H<sub>2</sub>O to get a final concentration of 2% (w/v). The gel solution was mixed thoroughly using an overhead mechanical stirrer until complete dissolving had occurred. The polymer solution was dropped into 3% KCl by using Encapsulator with nozzle size 300 μm to form uniform gel beads. Alg-Car gel beads were hardened using KCl for 3 h [16].

### 2.5.9. Alginate-Carrageenan/CaCl<sub>2</sub> (Alg-Carr/CaCl<sub>2</sub>)

Sodium alginate/*k*-Carrageenan gel (Alg-Car) was prepared by dissolving Alg/Car (1:1 w: w) in distilled H<sub>2</sub>O to get a final concentration of 2% (w/v). The gel solution was mixed thoroughly using an overhead mechanical stirrer until complete dissolving had occurred. The polymer solution was dropped into 2% calcium chloride (CaCl<sub>2</sub>) by using Encapsulator with nozzle size 300 μm to form uniform gel beads. The Alg-Car gel beads were hardened using CaCl<sub>2</sub> for 3 h [16].

### 2.5.10. Alginate-Carrageenan/KCl.CaCl<sub>2</sub> (Alg-Car/KCl.CaCl<sub>2</sub>)

Sodium alginate/*k*-Carrageenan gel (Alg-Car) was prepared by dissolving Alg/Car (1:1 w: w) in distilled H<sub>2</sub>O to get a final

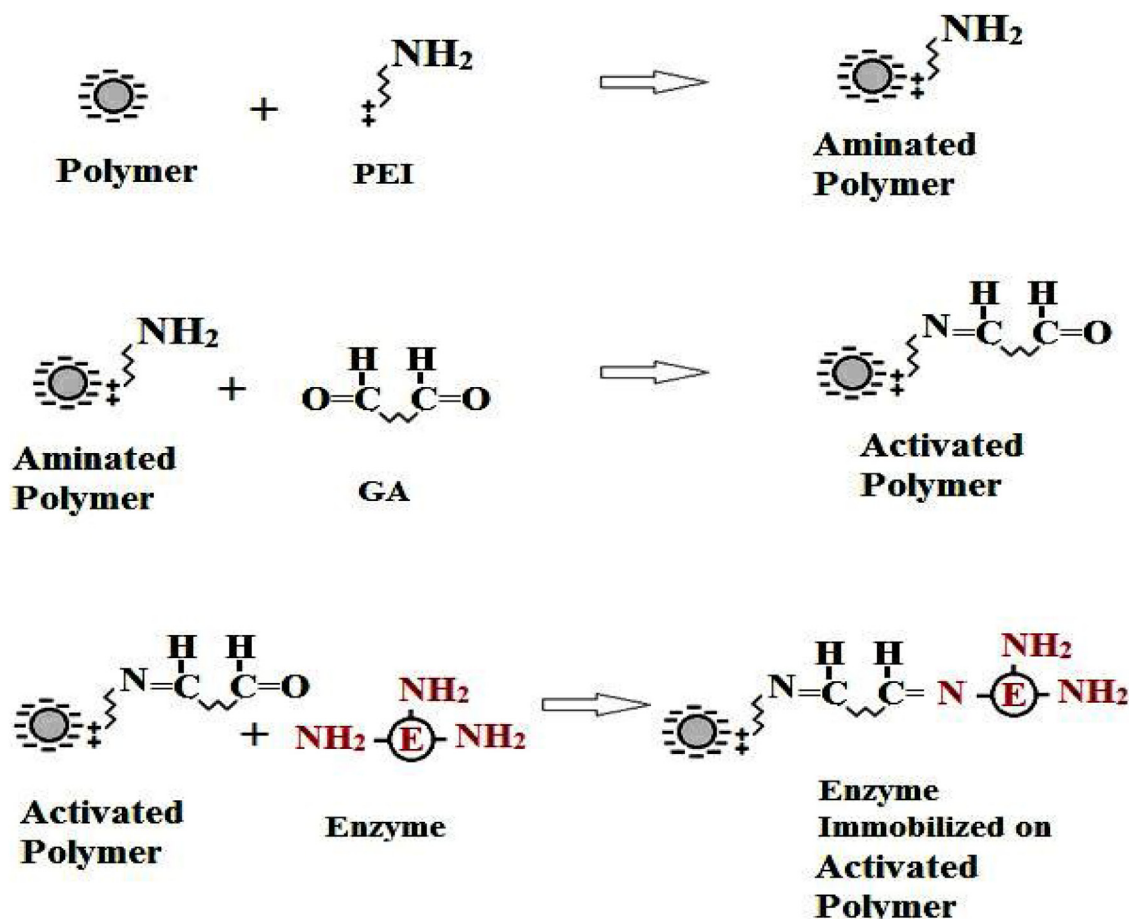
concentration of 2% (w/v). The gel solution was mixed thoroughly using an overhead mechanical stirrer until complete dissolving had occurred. The polymer solution was dropped into 3 % mixture solution of CaCl<sub>2</sub> and KCl in 1:1 ratio by using Encapsulator with nozzle size 300 μm to form uniform gel beads. The Alg-Car gel beads were hardened using CaCl<sub>2</sub> and KCl for 3 h.

### 2.5.11. Chitosan-Magnetic nanoparticles/NaOH (Ch-MNP/OH)

Chitosan (2 g) was dissolved in 100 ml distilled H<sub>2</sub>O and mixed with a solution of magnetic nanoparticles (Fe<sub>3</sub>O<sub>4</sub>). Then, spray the polymer solution into cross-linking solution of NaOH (5% w/v) through a nozzle of 300 μm using the Inotech Encapsulator. The prepared beads were hardened in crosslinking solutions for 3 h [15].

## 2.6. Beads modification (activation) for covalent immobilization

To modify the gel beads for covalent immobilization, the generated beads of all carriers were soaked in 4 % polyethyleneimine (PEI) solution (pH 9.5) for 3 h. The unreacted PEI was then removed from the beads by successive washing with distilled H<sub>2</sub>O. After washing, aminated gel beads were soaked in glutaraldehyde (GA 2.5 %) for 3 h to incorporate the new functionality aldehyde group. The gel beads were washed with distilled H<sub>2</sub>O to remove the unreacted GA. After that, the activated gel beads were ready for immobilization step as shown in [Scheme 1](#) for covalent immobilization of enzyme. Central composite design (CCD) was used to determine the optimum level of important factors for modification of the potent carrier (PEI %, PEI activation time, GA % and GA activation time).



**Scheme 1.** Mechanism of polymeric gel beads activation and enzyme immobilization.

## 2.7. Enzyme immobilization

$\alpha$ -amylase was immobilized on different polymers by covalent binding as described in Scheme 1. This was performed by mixing 2 ml of the partially purified  $\alpha$ -amylase (200 U) with 1 g of each prepared carrier. The mixture was left overnight at 4 °C, and then the beads were washed twice with distilled H<sub>2</sub>O and were used for immobilized  $\alpha$ -amylase assay. The best carrier that exhibited highest immobilization yield (IY %) and immobilization efficiency (IE %) was chosen and used through this study. IY (%) and IE (%) were calculated according to [17] as following:

$$IY (\%) = I / (A - B) \times 100 \quad (1)$$

$$IE (\%) = (I/A) \times 100 \quad (2)$$

Where: I is the total activity of immobilized enzyme, A is the total activity offered for immobilization and B is the total activity of unbounded enzyme.

## 2.8. Carrier characterization

### 2.8.1. Scanning electron microscope (SEM)

Morphological examinations on the surface of different carrier formulations, Ch-MNP, Ch-MNP/PEI, Ch-MNP/PEI/GA and Ch-MNP/PEI/GA/Enzyme were carried out using scanning electron microscopy (SEM, Quanta 250 FEG, accelerating voltage 200 V- 30 kV, FEI Company, Thermo Fisher Scientific). These investigations were performed in order to describe the morphological changes that happened on the surface after each reaction.

### 2.8.2. Fourier transforms infrared (FTIR) spectroscopy analysis

The FTIR absorption spectra of Ch-MNP, Ch-MNP/PEI, Ch-MNP/PEI/GA, Ch-MNP/PEI/GA/Enz and partially purified  $\alpha$ -amylase were measured by FTIR spectroscopy attenuated total reflection (ATR) mode Bruker VERTEX 70/70v model using the KBr disc technique. The aim of the test was to demonstrate the presence of the new functional group and carbonyl group formed at all different formulas. To do this, 2% (w/w) of the sample was mixed with dry KBr. The mixture was ground into a fine powder using an agate mortar before it was compressed into a KBr disc under a hydraulic press at 10,000 psi. Each KBr disk was scanned over a wave number range of 400–4000 cm<sup>-1</sup>, with a resolution of 4 cm<sup>-1</sup> and the characteristic peaks were recorded.

## 2.9. Characterization of free and immobilized $\alpha$ -amylase enzyme

### 2.9.1. Effect of temperature on $\alpha$ -amylase activity

The optimum temperature of the free and immobilized enzyme was determined by measuring the activity at different temperatures ranging from 20 to 70 °C in 0.1 M sodium phosphate buffer pH 7.0 and enzyme assay was performed. The activity of control temperature was taken as 100 % and the relative activity at each temperature was expressed comparing with the control. The activation energy ( $E_a$ ) of both free and immobilized  $\alpha$ -amylase was calculated from the slope of the Arrhenius plot according to the following equation:

$$\text{Slope} = - E_a / 2.303 R \quad (3)$$

Where: R is gas constant (8.314 KJ/mol).

### 2.9.2. Effect of pH on $\alpha$ -amylase activity

The effect of pH on enzyme activity (free and immobilized) was examined by performing the enzyme assay at different pHs ranging from 5.0 to 11.0 by using 0.1 M of the following buffer systems:

citrate-phosphate buffer (pH 5.0–6.0), sodium phosphate (pH 7.0–8.0) and glycine NaOH (pH 9.0–11.0) [18]. Enzyme activity was measured at optimum assay conditions and the relative activity was determined.

### 2.9.3. Effect of substrate concentrations (Determination of kinetic parameters)

For estimation of optimum substrate concentration for maximum enzymatic activity, enzyme activity (free and immobilized  $\alpha$ -amylase) was assayed at different starch concentrations (0.25–4 %) in 0.1 M sodium phosphate buffer (pH 8.0). The enzyme kinetic parameters, Michaelis–Menten constant ( $K_m$ ), and maximum reaction velocity ( $V_{max}$ ) were determined at optimum assay conditions according to [19] method. Also catalytic efficiency ( $V_{max}/K_m$ ) of  $\alpha$ -amylase was determined to demonstrate the affinity of enzyme with substrate.

### 2.9.4. Thermal stability

Thermal stability of the free and immobilized  $\alpha$ -amylase enzyme was determined by incubating the enzyme at various temperatures ranging from 50 to 80 °C for 60 min in the absence of substrate. Samples were taken at 15 min intervals and assayed for activity under optimized conditions. The residual activity was calculated as following by taking the enzyme activity at 0 min incubation as 100 %:

$$\text{Residual activity (RA\%)} = (\text{Final activity} / \text{Initial activity}) \times 100 \quad (4)$$

The thermal and thermodynamic constants were calculated as following according to [20].

Deactivation rate constant ( $K_d$ ) was calculated from the Arrhenius plot of Log RA (%) against time (min) using the following equation:

$$\text{Slope} = - K_d \quad (5)$$

The enzyme half-life time ( $t_{1/2}$ ) corresponds to the time period necessary for the residual activity to decrease by 50 % of its initial value and it was calculated from the following equation:

$$t_{1/2} = \ln 2 / K_d \quad (6)$$

Decimal reduction time ( $D$ -value) was defined as the time needed to reduce the initial activity by 90 % at a specific temperature and it was calculated as follows:

$$D\text{-value} = \ln 10 / K_d \quad (7)$$

The activation energy ( $E_d$ ) for  $\alpha$ -amylase denaturation was determined by a plot of log denaturation rate constants ( $\ln K_d$ ) versus reciprocal of the absolute temperature (K) using the following equation:

$$\text{Slope} = - E_d / R \quad (8)$$

The change in enthalpy ( $\Delta H^\circ$ , kJ/mol), free energy ( $\Delta G^\circ$  kJ/mol) and entropy ( $\Delta S^\circ$ , J/mol/K) for thermal denaturation of  $\alpha$ -amylase were determined using the following equations:

$$\Delta H^\circ = E_d - RT \quad (9)$$

$$\Delta G^\circ = -RT \ln (K_d \cdot h / k_B \cdot T) \quad (10)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ) / T \quad (11)$$

Where:  $E_d$  is the activation energy for denaturation (KJ/mol), R is the gas constant (8.314 J/mol/K), T is the absolute temperature (K),  $K_d$  is the deactivation rate constant (/min), h is the Planck constant ( $11.04 \times 10^{-36}$  Jmin) and  $k_B$  is the Boltzman constant ( $1.38 \times 10^{-23}$  JK).

### 2.9.5. pH stability

The pH stability of the free and immobilized  $\alpha$ -amylase was determined after pre-incubating the enzyme for 60 min at 30 °C with 0.1 M buffer systems at different pH range (pH 5.0–11.0) without substrate. The enzyme activity was measured at every 30 min intervals and the pH stability was expressed as RA % considering the initial enzyme activity at 0 min incubation as 100 %.

### 2.9.6. Operational stability (reusability)

The reusability of immobilized  $\alpha$ -amylase (Ch-MNP /PEI/GA/Enz) was studied for several cycles and the RA % was determined. After each cycle, the beads were washed to remove any residual substrate with sodium phosphate buffer (0.1 M, pH 8.0) and re-use to start a new cycle. The activity in the first run was taken as 100 % and the RA was expressed as a percentage of the starting operational activity.

### 2.9.7. Storage stability

The Immobilized enzyme was stored at 4 °C for 40 days and the residual activity was measured under the optimum conditions.

## 3. Results and discussion

### 3.1. Production and Partial purification of $\alpha$ -amylase enzyme

$\alpha$ -amylase produced by isolated bacterial strain *B. subtilis* MK1 (activity 141.63 U/ml and specific activity 4.8 U/mg) was partially purified by ethanol precipitation (data not shown). The fractions precipitated at 60 % ethanol possessed the highest  $\alpha$ -amylase specific activity (15 U/mg) which gave 3.1-fold purification. Therefore it was used for the preparation of the immobilized enzyme.

### 3.2. Enzyme immobilization

A novel method combining the advantages of physical adsorption and covalent binding methods was developed [17]. In this method, the enzyme was first adsorbed onto supports and the adsorbed enzyme was covalently cross-linked to the carriers.

The catalytic behavior of immobilized enzymes depends on the properties of their carriers. So,  $\alpha$ -amylase was immobilized on different carriers by covalent binding. The activated beads

(carriers) reacted with the enzyme as shown in Scheme 1. The reaction happened between the free C=O group located on GA and the NH<sub>2</sub> group found in the enzyme forming C=N- bond as reported by [15]. According to results introduced in Fig. 1, the most suitable carrier for  $\alpha$ -amylase immobilization was Ch-MNP/PEI/GA as it exhibited the highest IY of 55 %.

Polymeric nanocarrier can be fabricated easily in nanometer scale, in the range of 30 – 500 nm with a large surface area [21]. In addition they are attractive due to their simple synthesis, uniform size distribution, high stability, and abundance of functional groups for enzyme immobilization. The highest IY (81.6 %) using CCD was obtained at optimized conditions of 6% PEI, 4 h PEI activation time, 4% GA and 2 h GA activation time (data not shown). Optimization of beads modifications enhanced the IY by 1.5-fold compared with non-optimized.

### 3.3. Carrier characterization

#### 3.3.1. Scanning electron microscope (SEM) characterization

Scanning electron microscopy showed the modification which occurred during gel beads formation, activation and immobilization (Fig. 2). As shown, the Ch-MNP beads has rough surface that means high surface area available for immobilization. Noticeable changes were recognized along the steps of gel beads formation and accumulation of enzyme on the gel beads surface after immobilization. Moreover, a remarkable change in pore size can be recognized as the gel beads exhibited pores before treatment with PEI and GA. While after immobilization there was an accumulation of enzyme particles on the bead surfaces and the pores were decreased in size and tend to disappeared.

#### 3.3.2. FT-IR characterization

FT-IR spectroscopic analysis of Ch-MNP beads (A), aminated beads (Ch-MNP/PEI) (B), activated beads (Ch-MNP/PEI/GA) (C), immobilized beads (Ch-MNP/PEI/GA/Enz) (D) and partial purified enzyme (E) were carried out in a range varied from 400 – 4000 cm<sup>-1</sup> (Fig. 3). In this figure the five typical characteristic absorption bands of chitosan in accordance with the literature were appeared at 3428 cm<sup>-1</sup> (O-H and N-H stretching vibrations), 2921 cm<sup>-1</sup> (C-H stretching vibrations), 1656 cm<sup>-1</sup> (N-H bending vibrations) and 1073 cm<sup>-1</sup> (C-O-C stretching vibrations). The bands at 1320–1420 cm<sup>-1</sup> (C-N stretching vibrations) sharply increase in the spectrum of magnetic nanoparticles prepared in the presence of

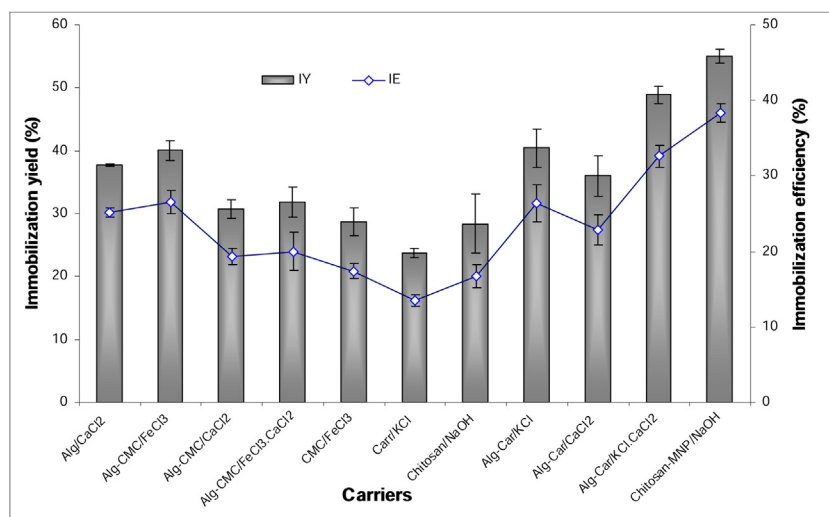
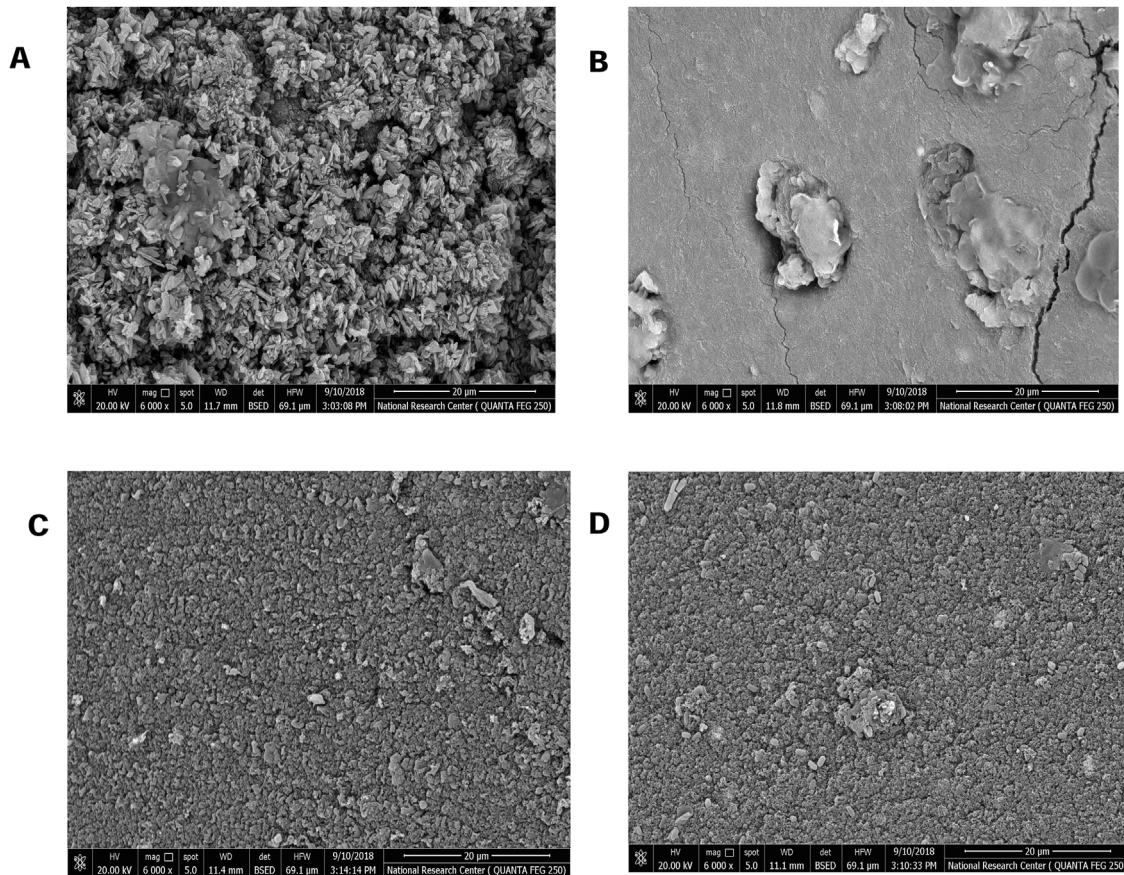
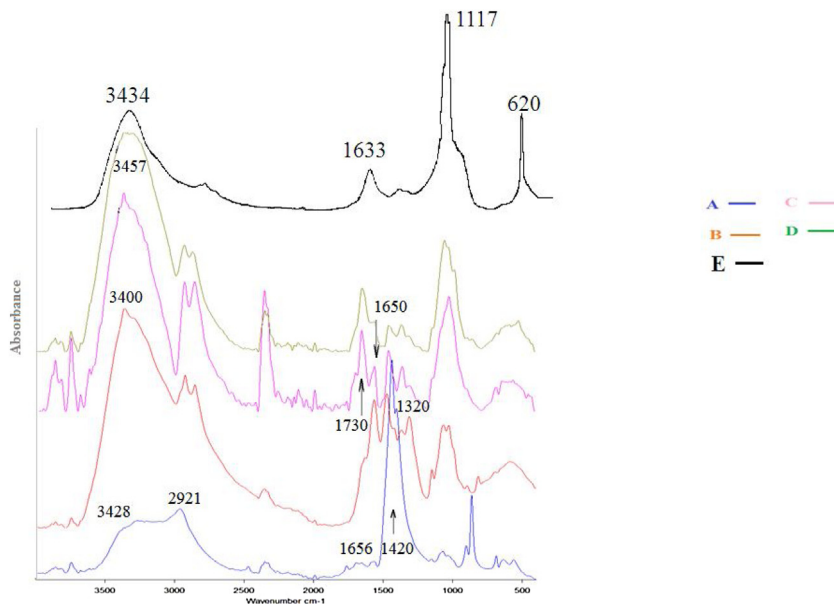


Fig. 1. Immobilization of *B. subtilis* strain- MK1  $\alpha$ -amylase on different carriers using covalent binding.



**Fig. 2.** Scanning electron microscope (SEM) showed the surface of different gel formulations of Ch-MNP beads (A), Ch-MNP/PEI (B), Ch-MNP/PEI/GA (C), Ch-MNP /PEI/GA/Enzyme (D).



**Fig. 3.** FT-IR spectroscopic analysis of Ch-MNP beads (A), Ch-MNP/PEI (B), Ch-MNP/PEI/GA (C), Ch-MNP /PEI/GA/Enzyme (D), Partially purified enzyme (E).

chitosan these characteristic bands indicate the obtaining of magnetic nanoparticles coated by chitosan (curve A). New broad peak at  $3400\text{ cm}^{-1}$  was appeared that corresponding to  $\text{NH}_2$  group, indicating the presence of amino group on the surface of beads as a result of amination process (curve B); on the other hand, the

activated beads showed two new peaks. One of them at  $1730\text{ cm}^{-1}$  corresponding to the  $(\text{C}=\text{O})$  group of GA free end, and the second one is at  $1650\text{ cm}^{-1}$  corresponding to the  $(\text{C}=\text{N}-)$  group that produced from the reaction with GA (curve C). Moreover, immobilized beads give broader peak at  $3457\text{ cm}^{-1}$ , pointed to increasing  $\text{NH}_2$  group's

concentration (curve D). When comparing with partial purified enzyme (curve E) we can notice that, the characteristic peaks of enzyme, at  $3434\text{ cm}^{-1}$  and at  $1117\text{ cm}^{-1}$ , are found in curve (D). From those data, it was revealed that the processes of amination, activation, and immobilization were successful. These results were in agreement with the results obtained by other published results [15].

### 3.4. Characterization of free and immobilized (Ch-MNP/PEI/GA/Enz) $\alpha$ -amylase

#### 3.4.1. Effect of temperature on $\alpha$ -amylase activity

According to the data observed from Fig. 4A, the optimum temperature was 45 and 55 °C, respectively for free and Ch-MNP/PEI/GA/Enz. Higher temperature (at 70 °C) decreased the activity by 42 and 11 %, respectively for free and Ch-MNP/PEI/GA/Enz. It is probably that binding of the carrier increased the rigidity, limited the conformational mobility of the enzyme molecules at high temperature, protecting it from denaturation [17,22]. In addition, it was thus proven that the immobilized enzyme could maintain its active structure even at a higher temperature [23]. Increased optimum temperature of immobilized  $\alpha$ -amylase makes it useful in biotechnological and industrial applications [24].

From the Arrhenius plot (Fig. 4B) the activation energy ( $E_a$ ) for the Ch-MNP/PEI/GA/Enz was 3.85 kJ/mol while that for free enzyme was 5.03 kJ/mol. This result suggested that, the immobilization promoted the enzyme catalytic efficiency by reducing the  $E_a$  wanted for the formation of activated enzyme-substrate complex [22].

#### 3.4.2. Effect of pH on $\alpha$ -amylase activity

The effect of pH on the activity of free and Ch-MNP/PEI/GA/Enz was illustrated in Fig. 5. The results showed that, there was an increase in  $\alpha$ -amylase activity with increase in pH value up to 8.0 for free and Ch-MNP/PEI/GA/Enz. However, the maximum activity of the free and Ch-MNP/PEI/GA/Enz occurs at the same pH, the Ch-MNP/PEI/GA/Enz exhibited a broader range of pH value (5.0–11.0). This report indicated that, the effective rate of the

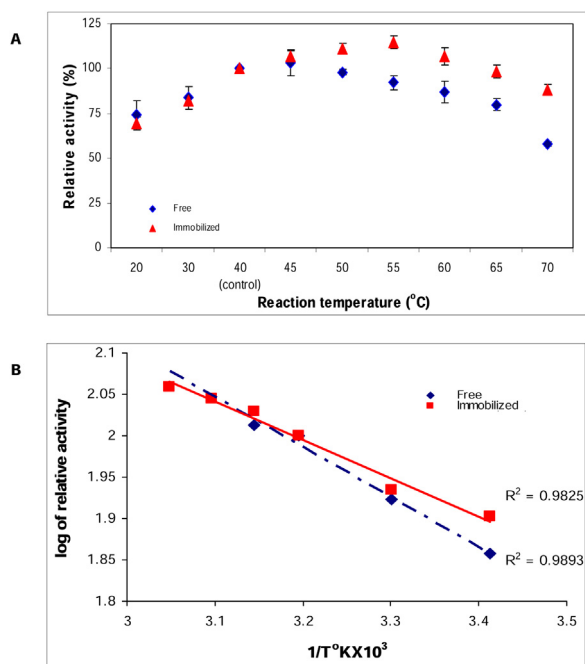


Fig. 4. Effect of different temperatures on  $\alpha$ -amylase activity (A) and Arrhenius plot for temperature dependence of the activity of free and immobilized  $\alpha$ -amylase (B).

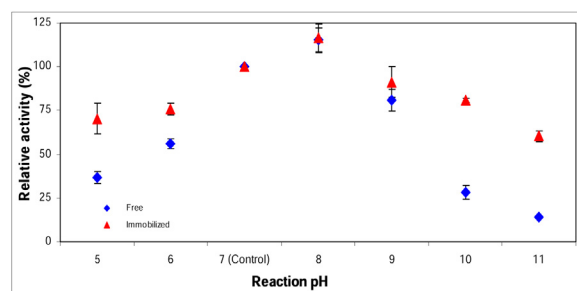


Fig. 5. Effect of pH on  $\alpha$ -amylase activity.

immobilized  $\alpha$ -amylase becomes less sensitive and has better adaptation to pH changes than the free enzyme. [25] reported that a detergent enzyme must withstand to harsh conditions such as high pH values (8.0–10.5). The higher relative activities might be attributed to the multipoint attachment of enzyme with  $\text{Fe}_3\text{O}_4$  at chitosan nanoparticles, which buffered variability of enzyme molecules when the pH was changed [17].

#### 3.4.3. Effect of substrate concentration on $\alpha$ -amylase activity

The activity of free and Ch-MNP/PEI/GA/Enz was estimated at various starch concentrations. The enzyme activity increased with increasing starch concentration up to 2 % for free enzyme and 2.5 % for Ch-MNP/PEI/GA/Enz (data not shown). The reaction kinetics of the free and Ch-MNP/PEI/GA/Enz was estimated from Hanes - Woolf plots under optimal conditions (Fig. 6). The results indicated that the calculated  $K_m$  value of the Ch-MNP/PEI/GA/Enz was 2.1-fold higher than the free form. In addition, the  $V_{max}$  of the Ch-MNP/PEI/GA/Enz was higher than the value of the free form by 1.2-fold. These effects might be a consequence of the changes in enzyme configuration upon immobilization. This pointed to increase the conversion rate of substrate to product but the affinity for substrate reduced which due to change in the microenvironment of the enzyme active site by decreasing structural flexibility of immobilized form. The  $V_{max}/K_m$  ratio for Ch-MNP/PEI/GA/Enz was 1.7-fold lower than the free enzyme. The results indicated that immobilized enzyme have less specificity to the substrate. [22] reported that a reduction in enzyme kinetic parameters after immobilization can be related to conformational changes in its structure.

#### 3.4.4. Thermal stability

The data presented in (Fig. 7A) explained the importance of immobilization process in enhancing the thermal stability of enzyme. The residual activity at 60 °C after 30 min and 60 min for the free enzyme was 41.5 % and 22.5 %, respectively and for the Ch-MNP/PEI/GA/Enz was 97.58 % and 93.54 %, respectively. At higher temperatures and longer times, the residual activity of the free enzyme declined dramatically. Obviously, increasing the

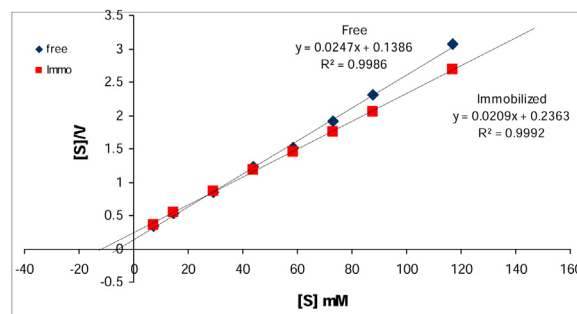


Fig. 6. Hanes - Woolf Plot of the free and immobilized  $\alpha$ -amylase.

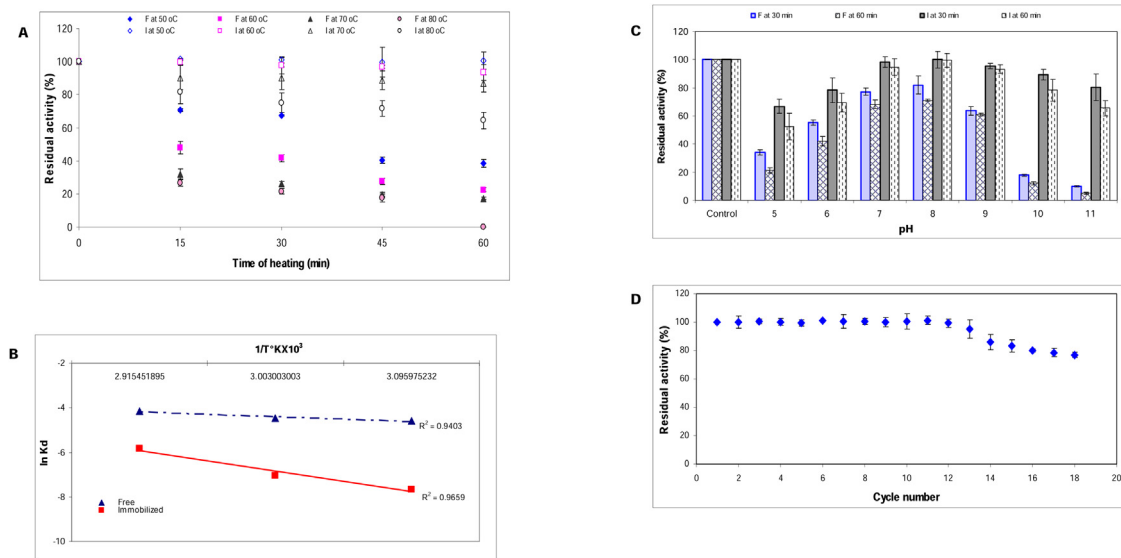


Fig. 7. Thermal stability (A), Arrhenius plot for energy of denaturation ( $E_d$ ) (B), pH stability (C) and operational stability (D) of  $\alpha$ -amylase enzyme.

thermal stability of Ch-MNP/PEI/GA/Enz demonstrated the successful role of immobilization method in protecting the enzyme against thermal inactivation and increasing its suitability for several industrial applications. This stability comes from the formation of high amount of covalent bonds has been presented to reduce enzyme mobility and conformational flexibility which in turn inhibit aggregation and unfolding of enzyme protein [20].

The heat inactivation profile of free and Ch-MNP/PEI/GA/Enz provides a relationship between the enzyme structure and function at a certain temperature (Table 1). Deactivation rate constant ( $K_d$ ) is an important parameter for the promote of an economic bioprocess at industrial level. With increasing temperature, the  $t_{1/2}$  decreased, the  $D$ -value decreased and the  $K_d$  increased (Table 1). At 60 °C, a 21.8-fold increase in  $t_{1/2}$  value of  $\alpha$ -amylase after immobilization has been observed. In addition, at 70 °C,  $D$ -value of Ch-MNP/PEI/GA/Enz calculated as 2642.1 min, which is higher than its free form by 13.3-fold. Increasing the  $t_{1/2}$  value and  $D$ -value confirm its improved thermal stability after immobilization [22].

The activation energy for denaturation ( $E_d$ ) is the lowest amount of energy wanted to initiate the denaturation process of enzyme (Fig. 7B). The results indicated that the required energy to denature Ch-MNP/PEI/GA/Enz was 3.8-fold higher than that for free enzyme.

The thermodynamic parameters as enthalpy ( $\Delta H^\circ$ ), Gibbs free energy ( $\Delta G^\circ$ ), and entropy ( $\Delta S^\circ$ ) were determined for free  $\alpha$ -amylase and Ch-MNP/PEI/GA/Enz. As seen from the results (Table 1), with increasing temperature there was a gradual decrease in  $\Delta H^\circ$  for both forms of enzyme. The  $\Delta H^\circ$  is the total amount of energy wanted to deactivate the enzyme. At 80 °C,  $\Delta H^\circ$  for the Ch-MNP/PEI/GA/Enz was higher than that of the free form

by 56.6 KJ/mol. [26] reported that a positive and higher value of  $E_d$  and  $\Delta H^\circ$  gives an indication of high thermal stability of the enzyme.

Gibbs free energy is known as the index of the energy wanted to cross the activation energy barrier of reaction.  $\Delta G^\circ$  value for the immobilized enzyme is more than the free enzyme indicating that immobilized enzyme requires more energy than free enzyme for thermal inactivation [8]. As shown in Table 1, there was an increase in the  $\Delta G^\circ$  value by 8.4 and 7.2 KJ/mol for Ch-MNP/PEI/GA/Enz over free enzyme at 60 and 70 °C, respectively.

The Entropy ( $\Delta S^\circ$ ) is the alteration in structural disorder upon protein denaturing and is directly related to enzyme stability [20]. The obtained  $\Delta S^\circ$  values were negative, meaning that the randomness or disorder decreasing in the transition from the native state to the denatured state of the free and immobilized enzyme [27] Zaboli et al. [8]. reported that the negative  $\Delta S^\circ$  values for the immobilized enzyme indicate the lack of enzyme aggregation during thermal inactivation. Decreasing  $\Delta S^\circ$  after immobilization points to high difference in entropy between the native state and the transition state may be due to the stabilization of enzyme conformation [28].

#### 3.4.5. pH stability

The pH stability for the free and Ch-MNP/PEI/GA/Enz was studied and displayed in (Fig. 7C). As seen, Ch-MNP/PEI/GA/Enz was significantly more stable than the free enzyme at all tested pH values especially at alkaline range. The Ch-MNP/PEI/GA/Enz exhibited great stability and kept 93.2 % and 78.3 % of its initial activity after 60 min at pH 9 and 10 while, the free enzyme preserved 61 % and 12 %, respectively. Furthermore, the residual activity of Ch-MNP/PEI/GA/Enz at pH 11 was 65.6 % after 60 min

Table 1  
Thermal inactivation kinetics and thermodynamic parameters of free and Ch-MNP /PEI/GA/Enzym.

Enzyme	$E_d$ (KJ/mol)	Thermal inactivation parameters	Temperature (°C)		Thermodynamic parameters	Temperature (°C)	
			60	70		60	70
Free	23.42	$K_d$ ( $\text{min}^{-1}$ )	$10.25 \times 10^{-3}$	$11.55 \times 10^{-3}$	$\Delta H^\circ$ (kJ/mol)	20.65	20.57
		$t_{1/2}$ (min)	67.60	59.99	$\Delta G^\circ$ (kJ/mol)	92.56	95.09
		$D$ -value (min)	224.56	199.30	$\Delta S^\circ$ (J/mol $^\circ$ K)	-0.22	-0.22
		$K_d$ ( $\text{min}^{-1}$ )	$0.47 \times 10^{-3}$	$0.87 \times 10^{-3}$	$\Delta H^\circ$ (kJ/mol)	86.21	86.13
Immobilized	88.98	$K_d$ ( $\text{min}^{-1}$ )	$0.47 \times 10^{-3}$	$0.87 \times 10^{-3}$	$\Delta G^\circ$ (kJ/mol)	100.91	102.31
		$t_{1/2}$ (min)	1472.98	795.34	$\Delta S^\circ$ (J/mol $^\circ$ K)	-0.05	-0.05
		$D$ -value (min)	4893.15	2642.05			



whereas the free enzyme lost 95 % of its activity. These results explained that, the immobilization process enhanced the stability and resistance of  $\alpha$ -amylase in both acidic and alkaline pHs. [21] reported that immobilization of enzymes on nano-carriers results in stabilization of active site conformation.

#### 3.4.6. Operational stability of Ch-MNP/PEI/GA/ $\alpha$ -amylase

Immobilization processes are fundamental keys for managing reuse of the enzyme over a long period. The Ch-MNP/PEI/GA/Enz beads can be easily separated from its products and reused many times for hydrolysis of starch. As shown in Fig. 7D the Ch-MNP/PEI/GA/Enz could be reused for 15 consecutive cycles with 83.2 % residual activity. The loss in enzyme activity may be related to repetitive encountering of substrate to the active site of immobilized enzyme that affects the binding strength between carrier and immobilized  $\alpha$ -amylase [29]. Furthermore, the reuse of immobilized enzyme in the reaction might be cause inactivation and denaturation of the enzyme. Our result is superior to that obtained by [29] where immobilized  $\alpha$ -amylase was reused for 10 cycles with residual activity 50 %.

#### 3.4.7. Storage stability

The main driving force for enzyme immobilization is stabilization. Storage Ch-MNP/PEI/GA/Enz at 4 °C for 21 days retained approximately 100% of its initial activity (data not shown). Moreover, after 40 days Ch-MNP/PEI/GA/Enz retained 86.4 %, such observations show the improved stability of the immobilized biocatalysts for a long time. [8] suggested that the multipoint attachment of the carrier surface to the enzyme may enhance stabilization. In addition Misson et al. [21], reported that the nano-environment surrounding enzyme molecules may prevent enzyme from deactivation.

## 4. Conclusion

The study describes a thermostable  $\alpha$ -amylase from isolated strain *Bacillus subtilis* strain-MK1. The enzyme was covalently bonded to different polymer supports.  $\alpha$ -amylase was successfully immobilized on Chitosan-magnetic nanoparticles (Ch-MNP/PEI/GA) with highest IY%. SEM and FTIR studies confirm the linkage between Ch-MNP/PEI/GA and the enzyme. Using statistical analysis (CCD) for beads modification enhanced the IY by 1.5-fold. Based on the results, Ch-MNP/PEI/GA/Enz showed higher optimum temperature by 10 °C than the free one. Immobilization improved the quality of  $\alpha$ -amylase by decreasing the activation energy by 1.31-fold. Also, Ch-MNP/PEI/GA/Enz was 21.79 and 13.26 times more stable than the free one at 60 and 70 °C, respectively. The higher value of  $E_d$  for Ch-MNP/PEI/GA/Enz (3.8-fold) compared with free indicates that more energy is required to enzyme thermal denaturation. Compared to the free enzyme, the Ch-MNP/PEI/GA/Enz exhibited lower  $E_a$ , lower  $K_d$ , higher  $t_{1/2}$  and higher  $D$ -values. The calculated thermodynamic parameters  $\Delta H^\circ$ ,  $\Delta G^\circ$  and  $\Delta S^\circ$  demonstrated that covalent binding between enzyme and Ch-MNP/PEI/GA increased its thermal stability. Moreover, Ch-MNP/PEI/GA/Enz showed higher pH stability at different pH values (5–11) compared with free enzyme. The Ch-MNP/PEI/GA/Enz was successfully used in batch mode for 12 cycles for effective degradation of starch with about 100 % residual activity. In addition, Ch-MNP/PEI/GA/Enz retained 86.4 % after 40 days storage at 4 °C. The results demonstrate that immobilized biocatalyst has the potential for different applications of industrial processes under harsh conditions.

## Author agreement

The author agrees to publish the manuscript in Biotechnology Reports.

## Funding

This work was supported by the National Research Centre, Egypt.

## Declaration of Competing Interest

The authors declare no competing financial interest.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00443>.

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