JJBS

Volume 15, Number 4,December 2022
ISSN 1995-6673
Pages 649 – 657
https://doi.org/10.54319/jjbs/150414

Jordan Journal of Biological Sciences

Kinetic, Catalytic and Thermodynamic Properties of Immobilized *B.Circulans* 25 Milk Clotting Enzyme on Activated Chitosan Polymer and Its Ability to Form Milk Curds

Samia A. Ahmed*, Mohamed A. Abdel-Naby and Ahmed F. Abdel-Fattah

Chemistry of Natural and Microbial Products Department, National Research Centre, Dokki, Giza, Egypt

Received: October 16, 2021; Revised: January 8, 2022; Accepted: January 19, 2022

Abstract

Enzymes immobilization has been widely used to increase their shelf life which is essential for the world's industries. Therefore, milk clotting enzyme (MCE) from *Bacillus circulans* 25 was immobilized by covalent binding, ionic binding and entrapment methods using various carriers. MCE covalently immobilized on activated chitosan polymer with the bifunctional agent glutaraldehyde (Ch-MCE) exhibited highest immobilization yield (74.6 %) and highest retained activity (77 %). The effect of temperature and pH on the enzyme activity was evaluated, and the results showed that Ch-MCE had higher optimum reaction temperature by 10°C and higher optimum pH by 1.0. Compared to the native MCE, Ch-MCE exhibited lower activation energy by 1.4-fold. In addition, Ch-MCE exhibited higher half-life time, lower deactivation rate constant and higher energy for denaturation than the free enzyme. After immobilization, V_{max}, K_M, specificity constant, turnover number, and catalytic efficiency of the enzyme were significantly changed. Furthermore, thermodynamic parameters for denaturation (enthalpy, entropy and Gibbs free energy) confirmed that the immobilization improved the catalytic properties of MCE. The reusability of Ch-MCE was also assessed, and 90.4% of its activity was retained after 7 catalytic cycles, confirming its suitability for industrial applications. Immobilization helps to overcome the limitations of reducing MCE catalytic activity associated with changes in temperature, pH and inhibitors, which makes it useful in industrial applications and biotechnological processes.

Keywords: Milk clotting enzyme, immobilization, stability, thermodynamic, catalytic, kinetics, reusability.

1. Introduction

Microbial rennin is more acceptable in cheese production as an alternative to chymosin from newborn ruminants due to ethical problems and increased demand for cheese making (da Silva, 2017). Rennin acts in two stages for milk protein coagulation by specific hydrolysis of peptide bond (Phe_{105}-Met_{106}) of κ-casein (da Silva, 2017). Recently, there have been additional applications of proteases in dairy technology to accelerate the ripening of cheese which is the most complex and important process for the development of favorable flavor and texture (Afroz et al., 2015). Industrial applications of enzymes have been restricted by several factors such as the high cost, instability at high pH and temperature, and their availability in small amounts (Duman and Bayer, 2021). In addition, the use of soluble enzymes has some drawbacks that increase the consumption of enzymes as inactivation, reduction of catalytic stability and difficulty of removal from the mixtures. These problems can be overpowered by immobilizing the enzyme on insoluble solid support. Enzyme immobilization is considered an effective technique which not only stabilizes enzymes under operating conditions but also allows enables reuse and continuous use (Wehaidy et al., 2018). A proper enzyme immobilization is a powerful tool to improve enzymatic properties, such as resistance to drastic reaction conditions, enhanced enzyme activity, improvement of enzyme specificity, and may even be coupled to purification. Enzymes can be immobilized by adsorption, entrapment, covalent binding and ionic binding methods. However, immobilization by covalent binding is the most effective procedure in establishing enzymes and preventing enzyme leakage due to the formation of irreversible covalent bond between support and the enzyme (Eskandarloo and Abbaspourrad, 2018). Covalent binding consists of two steps: first one, activation of functional groups found on the support surface by a specific reagent as glutaraldehyde (GA); and the second, adding enzyme to form covalent bond with activated support. In the coupling reaction, these activated groups will react with strong electron donating nucleophiles, such as the amino group (NH_2) and functional groups of certain amino acids on the surface of most enzymes (such as carboxylic group (COOH) of aspartic acid, amino groups (NH_2) of lysine, hydroxyl group (OH) of serine, and sulfhydryl group (SH) of cysteine). Immobilization can be performed using different supports whose properties play an important role in enzyme behavior. Desired properties of the insoluble supports include low-cost, non-toxic, high surface area, reusable and good stability (Narwal et al., 2016). Since

* Corresponding author. e-mail: dr_sa_ahmed@yahoo.com.

there is no universal support suitable for all enzymes and all applications, it is important to examine different supports using different methods of enzyme immobilization. Chitosan is one of natural polymer (polysaccharides) derived from chitin by deacetylation process and has excellent biocompatibility, no toxicity, cheapness, high mechanical strength, and a susceptibility to chemical modifications (Salazar-Leyva et al., 2017; Yandri et al., 2020). One common approach for enzyme immobilization on chitosan is through multipoint covalent binding between the functional groups present on the surface of an activated chitosan by cross-linking agents such as GA and the surface functional groups of the enzyme protein. When an enzyme becomes immobilized through many surface residues on a rigid support through very short spacer arms, important stabilizing effects may be achieved. Thermodynamics act as a key tool to understand the thermal deactivation process. Estimation of the thermodynamic parameters of the enzyme as enthalpy (ΔH^*), entropy (ΔS^*), and the Gibbs free energy (ΔG^*) can provide useful information as enzyme behavior, activity and thermostability. The suitability of enzymes for industrial application is judged by their thermodynamic parameters (Zaboli et al., 2019). Using free rennet to make cheese is not successful due to enzyme loss in whey, poor enzyme distribution, low yield, and poor cheese quality. So, the present study reports the immobilization of *B.circulans* 25 MCE and characterization of its catalytic, kinetics and thermodynamic parameters. Finally, evaluate the ability and reusability of Ch-MCE to form milk curds.

2. Materials and methods

2.1. Materials

Chitosan, Chitin, Dowex 1x4, Dowex 50W, Amberlite IR-120, Alumina, DEAE- sephadex A-25 and DEAE-cellulose DE-52 were obtained from Sigma Chemical Co., USA. Sodium alginate was supplied from BDH Chemical Ltd., Poole, England. Ceramic, wool and chicken bones were collected from the local market in Egypt. Skim milk powder spray dried (heat treated grade) was made in USA and obtained from the Ministry of Agriculture, Giza. Egypt. Other chemical reagents used were of analytical grade.

2.2. Methods

2.2.1. Enzyme production

MCE from *B.circulans* 25 has been produced according to the previous work (Ahmed et al., 2018). The medium used for MCE production had the following composition (g/L): lactose 20, yeast extract 1, peptone 1, K_2HPO_4 2 and $MgSO_4.7H_2O$ 0.25, and the pH was adjusted to 6.0 prior to sterilization. One mL of cell suspension of 24 h-old slant (OD600~0.3) was transferred to 50 mL sterile medium in 250-mL Erlenmeyer flask. The flasks were incubated at 35°C and 180 rpm for 24 h. The broth media after incubation was centrifuged at 6000 x g and 4°C for 15 min and the cell free filtrate was considered as source of crude enzyme.

2.2.2. Milk clotting activity and protein determination

Milk clotting activity was estimated according to Narwal et al. (2016) method. Enzyme solution (2.5 mL) or certain weight of Ch-MCE was incubated with 10 mL skim milk (12 g dry skim milk/100 mL of 0.01 M $CaCl_2$) at 40°C. The time at which the first particles were formed was recorded. The time at which the first particles were formed was recorded by a stopwatch. One unit of the MCE activity (U) was equalized to 10 mL milk clotted within 10 min. The protein content of the MCE preparation was estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. The amount of immobilized protein was calculated by subtracting the amount of unbound protein from the amount of protein originally added for immobilization.

2.2.3. Enzyme immobilization

Immobilization yield (IY %) and immobilization efficiency (IE %) were calculated according to Abdella et al. (2020) to select the suitable carrier and method.

$$\text{Immobilization yield (\%)} = I/(A–B)\ X100 \quad (1)$$

$$\text{Immobilization efficiency (\%)} = (I/A)\ X\ 100 \quad (2)$$

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

2.2.3.1. Immobilization of MCE by covalent- binding

Chitosan beads were prepared by shaking 0.4 g chitosan in 5 mL of 0.01 M HCl containing GA (2.5 %) at 30°C for 2 h. The beads were precipitated using 0.1 N NaOH, collected by filtration, and washed with distilled H_2O (to remove the excess GA). Then 5 mL enzyme solution (440U) was mixed with the wet beads by gently shaking. After 2 h at 30°C, the unbounded enzyme was removed by washing with distilled H_2O until no activity was detected. One gram of other carriers (chitin, wool, chicken bone, As-alumna, ceramic or PVC) was shaken in 25 mL Tris–HCl buffer (0.01 M, pH 6.0) containing 2.5 % GA at 30°C for 2 h. The carriers were filtered off, and washed with distilled H_2O to remove the excess GA. Then each treated carrier was incubated with Tris HCl buffer (5 mL, 220 U of MCE). After incubation at 30°C for 2 h, the unbounded enzyme was removed by washing with distilled H_2O (Abdel-Naby et al., 1998).

2.2.3.2. Immobilization of MCE by ionic- binding

The anion or cation exchanger (1 g) equilibrated with phosphate buffer (0.1 M, pH 6.0) or Tris-HCl (0.1 M, pH 8.0) and was incubated for 16 h at 4°C with certain volume of the enzyme solution (50 U) in the same buffer (Eskandarloo and Abbaspourrad, 2018). The unbounded enzyme was removed by washing with the same buffer.

2.2.3.3. Immobilization of MCE by alginate entrapment

In this experiment, 10 mL of different concentrations of Na-alginate solution were mixed with equal volume of enzyme solution (200 U) to obtain final concentration range of 2-8 % (w/v). The whole mixture obtained by sodium alginate was extruded drop wise through a Pasteur pipette into a gently stirred 0.1 M $CaCl_2$ solution for 2h. The resulting beads with a diameter of ~1.0-1.5 mm were collected, washed with buffer, and kept for 24 h at 4°C to remove the unbound enzyme (Dey et al., 2003).

2.2.4. Characterization of free and chitosan immobilized MCE

2.2.4.1. Optimum pH and pH stability

The effect of pH on the activity of free and Ch-MCE was investigated in 0.01 M buffer with different pH values (4.5 – 8.5). The relative activity was calculated according to Eq.3.

Relative activity (%) = (A1/A2) X 100 (3)

Where: A1 (activity detected under the certain condition and A2 (activity detected under the optimal condition).

The stability to pH was investigated by pre-incubating enzyme samples in 0.01M tris - HCl buffer with pH ranging from 5.0 to 9.0 at 25°C for 1h followed by adjusting the pH to the optimal of each enzyme form. The residual (retained) activity was assayed under the standard conditions and calculated to according to Eq.4.

Residual activity (%) = (Af / Ai) × 100 (4)

Where: Af (final activity detected) and Ai (initial activity detected).

2.2.4.2. Optimum temperature and thermal stability

The enzyme samples in 0.01M tris - HCl buffer at pH 6.0 and 7.0 (for free and Ch-MCE), respectively were subjected to different temperatures (from 30°C to 100°C). The activation energy (E_a) was estimated from the slope of Arrhenius plot of log the residual enzyme activity (%) against reciprocal of absolute temperature in Kelvin (°K) according to Eq.5.

Slope = - E_a / 2.303 R (5)

Where: E_a (activation energy) and R (gas constant 1.976 Kcal/ mol).

Temperature coefficient value (Q_{10}), the rate of an enzymatic catalysis reaction changes for every 10 °C rise in temperature, was calculated as reported by Wehaidy et al. (2018) as Eq.6.

Q_{10}= antilog E = (E x10/RT^2) (6)

Where $E = E_a$ = activation energy

For thermal stability, free and Ch-MCE were heated at different temperatures (40–80°C) in the absence of substrate for different time intervals (15–120 min). Every 15 min, a sample was removed and the residual activity was estimated under standard assay conditions. The enzyme activity without heating was taken as 100%. Deactivation rate constant (k_d) was determined according to Eq.7 from the semi logarithmic plot of residual activity (%) versus time (min) (Singh et al., 2019).

Slope = - k_d (7)

Half-life ($t_{1/2}$) value of inactivation is given according to Eq.8.

$t_{1/2}$= ln2/ k_d (8)

The energy for denaturation of enzyme (E_{ad}) was calculated from Arrhenius plot of (ln k_d) as a function of (1/T) temperature in Kelvin (°K) using the following in Eq.9.

Slope = $-E_{ad}$/ R (9)

2.2.4.3. 2.2.4.3. Effect of substrate concentration

Both the free and Ch-MCE activities were assayed with different substrate concentrations ranged from 1 to 12 % (w/v) at optimal assay conditions. Michaelis–Menten constant (K_M) and maximum velocity (V_{max}) were estimated from Lineweaver and Burke (1934). In addition, the turnover number (k_{cat}), catalytic efficiency (k_{cat} / K_M), specificity constants

(V_{max} / K_M), free energy of substrate binding ($\Delta G^*_{E–S}$) and free energy of transition state binding ($\Delta G^*_{E–T}$) were estimated according to Wehaidy et al. (2018) as Eqs. (10-15).

k_{cat} = ($k_b T$ / h) x e $^{(-\Delta H^*/RT)\ x\ e\ (\Delta S^*/R)}$ (10)

Where: k_b (Boltzmann's constant =1.38×10^{-23} J/ K), T (absolute temperature (°K), h (Planck's constant =6.626×10^{-34} Js), N (Avogadro's number =6.02×10^{23}/ mol), and R (Gas constant=8.314 J/ K/ mol).

ΔH^* (Enthalpy) = $E_a - RT$ (11)

ΔG^* (Gibbs free energy of activation) = $-RT\ ln\ (k_{cat}\ h / k_b$ x $T)$ (12)

ΔS^* (Entropy) = ($\Delta H^* - \Delta G^*) / T$ (13)

$\Delta G^*_{E\text{-}S}$ (Free energy of substrate binding) = $-RT\ ln\ K_a$, where $K_a = 1/ K_M$ (14)

$\Delta G^*_{E\text{-}T}$ (Free energy for transition state formation) = $-RT\ ln\ (k_{cat} / K_M)$ (15)

2.2.4.4. Effect of metal ions

The metal ions ($ZnSO_4$, $CoCl_2$, $CaCl_2$, $MnSO_4$, $CuSO_4$, $MgSO_4$, and $HgCl_2$) were added individually (10 mM) to the reaction mixture. Both free and Ch-MCE activities were assayed under optimal assay conditions.

2.2.5. Suitability of Ch-MCE in making cheese (reusability)

A weight sample (4 g) of Ch-MCE (wet) was placed in a bag of muslin. The bag was immersed in skim milk solution (10 mL). The mixture was incubated at 85°C until forming the colt. At the end of the reaction, the bag containing Ch-MCE was removed from the colt, washed with H_2O, and re-suspended in a freshly prepared substrate (10 mL) to start a new run.

3. Result and discussion

The efficiency of enzyme immobilization was evaluated by different parameters including the residual activity (RA %), the immobilization efficiency (IE %), the loading efficiency LE (immobilized enzyme activity/gram carrier); however, the IY (%) is the key parameter that it represents the general output of the immobilization process efficiency.

3.1. Enzyme immobilization

3.1.1. Immobilization of MCE by covalent- binding

Immobilization of MCE by covalent-binding was achieved by cross-linking between the enzyme and activated carriers throughout GA as a spacer group. Abdella et al. (2020) explained that the interaction occurred between the NH_2 groups (in the enzyme protein molecule) and the free C=O group (located on GA) forming C=N– bond. In the case of chitosan, the used amount of MCE was higher than that used for other carriers due to its higher loading efficiency. The data presented in Table 1 indicated good IY and LE, especially with chitosan (74.6 % and 315.0 U/g carrier). Our result is higher than that obtained by Esposito et al. (2016) on immobilized MCE by 1.1-fold. Covalent binding is the best method regarding to the strength of the interactions, reduce protein leakage (due to the formation of stable cross- linking between the enzyme and the carrier via

spacer group). In addition, side chains of several amino acids can form covalent bonds with carriers (Nwagu et al., 2011). Moreover, covalent binding via spacer group may increase the local surface area and consequently reduced the protein crowding. Further, chitosan was preferred as a suitable carrier because it is cationic, biodegradable, inert, non-toxic and biocompatible compound (Hosseini and Varidi, 2021).

3.1.2. Immobilization of MCE by ionic- binding

A series of ion exchangers was used for the immobilization of MCE by ionic binding. The results in Table 2 showed that the most suitable ion exchanger for MCE immobilization is Dowex 50W with highest LE (19.0 U/g carrier) and highest IY (56.7 %). On the contrary, MCE immobilized on DEAE-Sephdex A-25 had no activity, which might be due to that the enzyme's active sites were involved in the fixation process. Similarly, Abdel-Naby et al. (1998) pointed to the low bound enzyme for protease immobilization by ionic binding.

Table 1. Immobilization of *B. circulans* 25 MCE by covalent-binding.

Carrier	Added enzyme (U/g)(A)	Un bounded enzyme (U/g)(B)	Immobilized enzyme (U/g)(I)	Immobilization yield (IY %)
Chitin	220.0	100.00	79.20	66.00
Wool	220.0	131.40	49.75	56.15
Chicken bones	220.0	133.30	57.75	66.61
Ceramic	220.0	122.40	51.06	52.31
As-alumina	220.0	118.32	60.16	59.17
PVC	220.0	131.92	54.89	62.32
Chitosan	440.0	17.60	315.00	74.57

Table 2. Immobilization of *B. circulans* 25 MCE by ionic-binding.

Carrier	Added enzyme (U/g)(A)	Un bounded enzyme (U/g)(B)	Immobilized enzyme (U/g)(I)	Immobilization yield (IY %)
Dowex 50 W	50.0	16.40	19.00	56.65
Dowex 1x4	50.0	17.10	8.50	25.84
Ambelite IR- 120	50.0	23.80	10.00	38.17
DEAE–Cellulose DE-52	50.0	39.80	3.30	32.35
DEAE – Sephadex A-25	50.0	26.30	-	-

3.1.3. Immobilization by alginate entrapment

The most common method to prepare hydrogels from an aqueous alginate solution is to combine the solution with ionic cross-linking agents, such as divalent cations (i.e., Ca^{2+}). MCE was immobilized by entrapment in Ca-alginate with different concentrations as presented in Table 3. The results showed that the LE was decreased from 37.2 to 18 U/10 mL gel with the gel concentration increased from 2 to 8 %. This is probably due to the decrease of the gel porosity with the increase of Na-alginate concentration, and consequently the diffusion limitation was developed. Similar observation was previously reported for entrapped proteases (Abdel-Naby et al., 1998; Lamas et al., 2001). Hosseini and Varidi (2021) reported that decreasing alginate concentration from 0.2 to 0.04% increased the rennet encapsulation efficiency. The pore size of the gel, reflected in the viscosity of the carrier due to the size of the molecule and/or its concentration, can affect the diffusion of substrates or products and limit the reaction rates of the entrapped enzyme. Narwal et al. (2016) immobilized bacterial MCE in alginate-pectate interwoven gel with higher IY (73%).

Table 3. Immobilization of *B. circulans* 25 MCE by entrapment in Ca-alginate.

Na – alginate concentration (%)	Added enzyme (U/10ml gel) (A)	Un bounded enzyme (U/10ml gel) (B)	Immobilized enzyme (U/10ml gel) (I)	Immobilization Yield (IY %)
2	200.0	9.00	37.20	19.48
4	200.0	11.60	24.30	12.89
6	200.0	13.90	21.10	11.34
8	200.0	15.30	18.00	9.74

3.2. Characterization of free and Ch-MCE

Among all preparations, chitosan- immobilized MCE (Ch-MCE) gives the highest IY and the highest LE, consequently, in the following experiments Ch-MCE was used. Chitosan is a versatile, easily easily-to- process cationic biomaterial that can be widely used in many applications such as medical, tissue engineering, antibacterial dressings, and enzyme immobilization, due to its biological and chemical properties such as biodegradability, safety, and non-toxicity (El-Shishtawy et al., 2021; Hosseini and Varidi, 2021). Much scientific literature reported that in many cases of enzyme immobilization methods the protocol leads to losses in some enzyme activity. Ch-MCE retained 77 % of the initial specific activity shown by the free enzyme. The enzyme activity may decrease due to the corresponding changes caused by the immobilization (Siar et al., 2017). Also, the decrease in the specific activity may be due to the diffusional limitation of the substrate and product flow. Although GA creates very strong bonds between enzymes and carriers, it changes the structure of some enzymes during the binding process, causing loss of their activity (Salazar-Leyva et al., 2017).

3.2.1. Effect of pH and pH stability of the free and Ch-MCE

The pH can either change the ionization of the enzyme-substrate complex or alter the protein structure of the enzyme. As illustrated in Figure 1, the immobilization shifted the optimum activity of the MCE from free 6.0 to 7.0. MCE. At higher pH values up to 8.0, the drop in activity was more pronounced with the free than that of the Ch-MCE. These effects may be due to the changes of the ionic microenvironment of the enzyme active site and /or distribution of the surface charges of the carrier after immobilization (Talbert and Goddard, 2012;

Pervez et al., 2017). These are due to the use of cationic carrier like chitosan which interacts with anionic groups on the enzyme surface. Pervez et al. (2017) reported that the pH in the immediate vicinity of enzyme molecule may change, depending upon the surface and residual charge on the solid support (chitosan is positively charged due to amino groups).

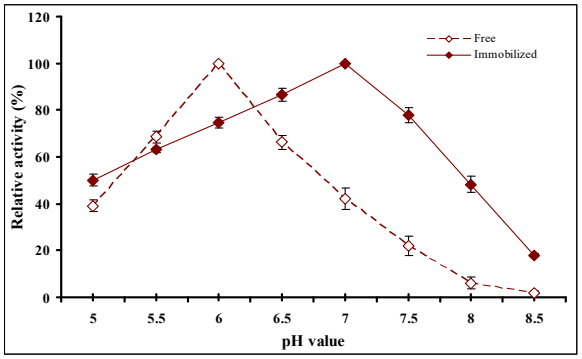

Figure 1. Effect of pH on the activity of free MCE and Ch-MCE.

The results in Figure 2 showed that the pH stability of Ch-MCE was shifted to higher pH values compared to the free MCE. These results reveal the same trend as in the shift of the optimum pH. The results showed that covalent binding maintained the MCE catalytic activity especially in the case of alkalinity. Pervez et al. (2017) reported that immobilization decreases the inhibition of enzymes either by stabilizing the enzyme structure or by eliminating the inhibitor. Moreover, multipoint covalent attachment between the enzyme molecule and an activated carrier makes the structure of the enzyme more rigid, consequently decreasing conformational changes which can be induced by extreme pH (Salazar-Leyva et al., 2017). Narwal et al. (2016) suggested that titratable acidity of milk should be between 0.19 to 0.25 % lactic acid equivalents (which correspond to pH from 5.0 to 6.5) at the time of adding an enzyme. Therefore, for industrial applications, the MCE should be stable between these pH ranges.

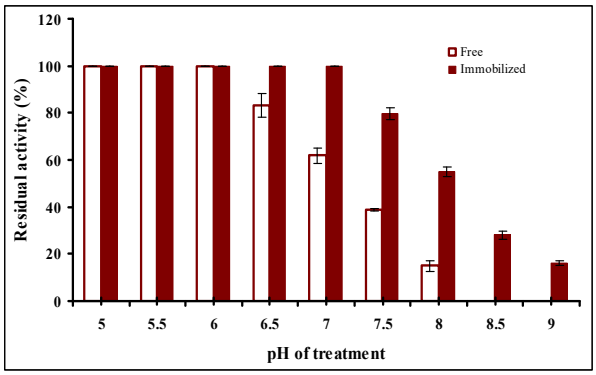

Figure 2. pH stability of free MCE and Ch-MCE.

3.2.2. Effect of temperature and thermal stability of the free and Ch-MCE

As seen in Figure 3, immobilization increases the optimum temperature by 10°C which is probably a result of the thermal stability enhanced by immobilization. Covalent binding of enzyme onto chitosan enhanced the optimum temperature and stability of the biocatalysts for thermal inhibition (Salazar-Leyva et al., 2017). Hosseini and Varidi (2021) reported that free and immobilized rennet showed the highest activity at the same temperature (40 °C), and at higher temperatures there is a sharp decrease in the activity of the free enzyme compared to the immobilized enzyme.

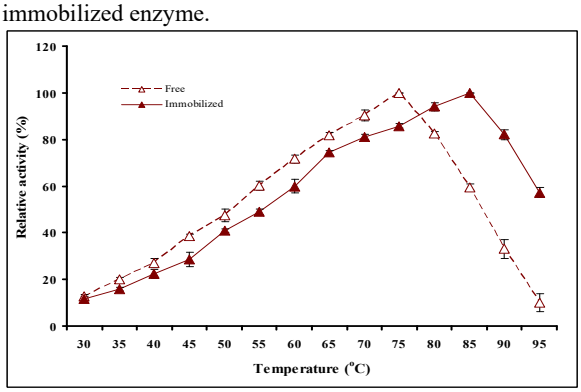

Figure 3. Effect of temperature on the activity of free MCE and Ch-MCE.

The calculated E_a (Figure 4) indicated that the energy required to form the enzyme-substrate complex for the free enzyme (36.76 kJ/mol) was 1.4-fold higher than that required for the Ch-MCE (34.31 kJ/mol), confirming that immobilization improved the catalytic efficiency of the enzyme by lowering the energy required to make the activated complex of enzyme and substrate (Wehaidy et al., 2018). This result of E_a was lower by 1.7-fold than that obtained for immobilized *Aspergillus fumigatus* protease (Hernandez-Mariinez et al., 2011).

Similar decrease in E_a was reported by El-Shishtawy et al. (2021) for the immobilization of catalase enzyme. Reduced E_a of the immobilized enzyme compared to free enzyme may be due to the mass transfer limitations (Thakrar and Singh, 2019).

Although thermostable enzymes are more suitable for industrial applications than mesophilic enzymes, stability of MCEs for long times at mild temperatures is very important for their suitability in making cheese. The results in Figure 5 showed that the immobilization improved the stability of MCE to the thermal inhibition. After heating at 60°C, the Ch-MCE was stable up to 120 min with 100 % residual activity whereas, the free MCE lost about 90.4 % of its initial activity. In addition, after heat treatment at 70°C for 90 min, the free MCE was completely inhibited; however, the Ch-MCE retained 40 % of its initial activity. The increase in enzyme stability after immobilization was possibly related to the higher rigidity of the immobilized form (Yang et al., 2017). The immobilized enzyme would be bound to the support via multipoint covalent bonds, and thus its structure would be rigid, and therefore it would be more resistant to the thermally induced denaturations which would reduce free enzyme activity (Wahba et al., 2021). In addition, the stability to heat inhibition enhancement after immobilization could be caused by the carrier that protects the enzyme from denaturation by absorbing a great amount of heat (Figueira et al., 2011).

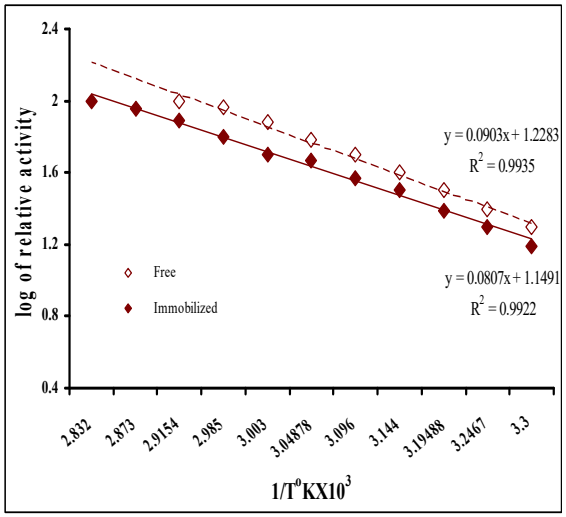

Figure 4. Arrhenius plot for temperature dependence of the activity of free MCE and Ch-MCE.

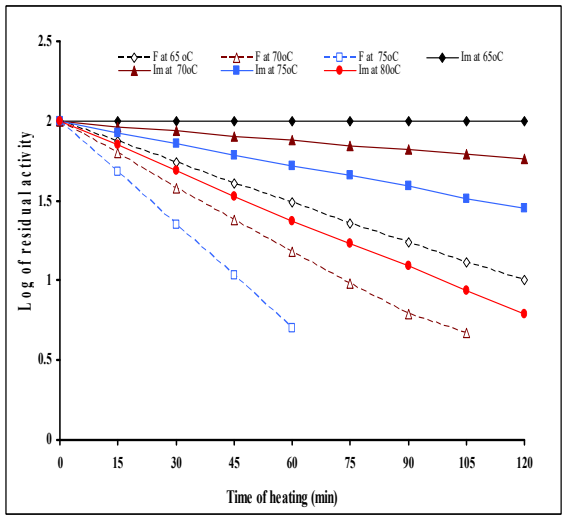

Figure 5. First-order plots of the effect of thermal inactivation of free MCE and Ch-MCE.

The calculated k_d in Table 4 indicated that the stability of the Ch-MCE to the thermal inhibition was superior to that of the free MCE (the lower k_d, the more thermo stable enzyme). For example, k_d at 70°C for the free enzyme was higher by 3-fold than that of the Ch-MCE. These results confirm the effectiveness of the MCE immobilization on chitosan for increasing the thermal stability. The decrease of k_d value is predicted because the condition of the enzyme is less flexible than in water, so the unfolding of the enzyme is decreased, and the stability of the enzyme has increased (Yandri et al., 2020). In addition, $t_{1/2}$ of the Ch-MCE at 65, and 75°C were higher by 6 and 5-fold, respectively than that of the free MCE. The binding between the enzyme and the carrier reduces conformational flexibility and thermal vibration, thus protecting the immobilized protein from denaturing and unfolding by increasing the temperature (Figueira et al., 2011).

Table 4. Thermal properties of free and Ch-MCE.

Property	Milk-clotting enzyme	
	Free	immobilized
Deactivation rate constant k_d / min, at		
60 °C	8.35 x 10^{-3}	Stable for 120 min
65 °C	13.5 x 10^{-3}	1.97 x 10^{-3}
70 °C	21.167 x 10^{-3}	4.45 x 10^{-3}
75 °C	Unstable	10.09 x 10^{-3}
Half life time $t_{½}$ (min), at		
60 °C	36.04	Stable for 120 min
65 °C	22.29	152.79
70 °C	14.22	66.88
75 °C	Unstable	29.83
Energy for denaturation E_{ad} (KJ/ mol)	217.34	378.73

The E_{ad} for Ch-MCE was greater by 161.39 kJ/ mol than that obtained for free MCE (Figure 6 and Table 4). This result indicated that immobilized form needed more energy for deactivation compared to the free form. The stability of enzymes is enhanced by the rigidification of their immobilized structure via the multipoint covalent linkages established between them and their supports (Wahba et al., 2021). The energy of thermal inactivation is an important factor to judge its thermal stability (Thakrar and Singh, 2019).

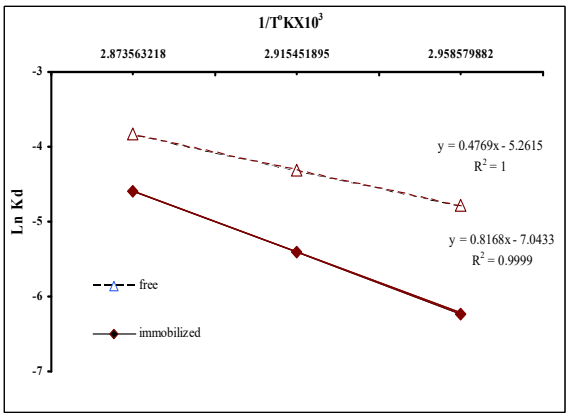

Figure 6. Arrhenius plot for activation energy of denaturation for free MCE and Ch-MCE.

3.2.3. *Effect of substrate concentration*

In order to determine the kinetic parameters of the MCE, the activity of the free and Ch-MCE was measured at different substrate concentrations and the Lineweaver-Burk was plotted. As seen in Figure 7, Ch-MCE provided K_M 1.3-fold higher and V_{max} 1.3-fold lower than the free MCE suggested that the main problem is just diffusion problems for the entry of the substrate inside the biocatalyst particle. Wahba et al. (2021) found that, the increase in K_M indicates that the affinity of an enzyme for its substrate is decreased due to the resistance to mass transfer and the reduction of the immobilized enzyme

flexibility. Furthermore, decreasing the affinity of the immobilized enzyme may be due to the changes in the enzyme structure by the immobilization process or the reduction of substrate access to the active site of the enzyme (Pervez et al., 2017; Hosseini and Varidi (2021). The decrease in V_{max} of immobilized enzymes could be attributed to the interactions between these enzymes and their respective supports which could reduce the flexibility of the enzymes (Wahba et al., 2021). In addition, the V_{max} / K_M of Ch-MCE was decreased by 1.6-fold compared to the free MCE (Table 5). Low V_{max} / K_M ratio specifies the smaller specificity of immobilized form for casein than the free MCE (Singh et al., 2019). Similar observations were reported by Cahyaningrum and Sianita (2014).

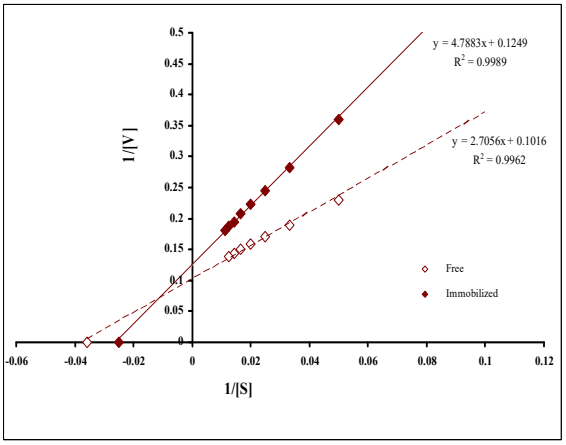

Figure 7. Lineweaver-Burk Plot for free MCE and Ch-MCE.

k_{cat} represents the maximum number of reactions catalyzed per minute. The k_{cat} of Ch-MCE was higher by 1.5-fold compared to the free MCE (Table 5). The k_{cat} / K_M of free and Ch-MCE were 0.154 and 0.174 /S mg/mL, respectively, indicating that the immobilization technique enhanced the catalytic efficiency ~ 13 %. On contrary, Mafra et al. (2019) found that the immobilized enzyme catalytic efficiency was 34 % lower than the free form. The recorded data in Table 5 showed a decrease in the Ch-MCE ΔH^* compared to the free MCE. In addition, ΔS^* for Ch-MCE was lower by 14.2 kJ/ mol than that of the free MCE. The lower ΔH^* and the negative ΔS^* values for Ch-MCE pointed to the stability and the effective transitional state of the complex of enzyme-substrate (Wehaidy et al., 2018). The ΔG^* of a substance results from the stabilizing forces present in protein's structure, such as Van der Waals interactions and hydrogen bonds. The higher ΔG^* is associated with more tolerance toward heat inactivation (Zaboli et al., 2019). As shown in Table 5, ΔG^* for the Ch-MCE was higher by 5.49 kJ/ mol than the free MCE indicating that a change of the Ch-MCE-substrate complex into products is less spontaneous in comparison with free MCE. The catalytic reaction can be evaluated by estimating ΔG^* for enzyme-substrate complex conversion into products (Riaz et al., 2007).

The ΔG^*_{E-T} for Ch-MCE was lower 1.0-fold compared to the free MCE. This result indicated that this reaction is more spontaneous for Ch-MCE than for free MCE (Ferreira et al., 2018). In addition, the ΔG^*_{E-S} confirmed that the Ch-MCE requires higher amount of free energy by 1.1-fold to form this transition state compared to the free MCE. The same behavior among soluble and conjugated MCE from *B. subtilis* KU710517 was reported by Wehaidy et al. (2018).

3.2.4. Effect of metal ions

The effects of various metal ions on the Ch-MCE activity compared to the free MCE were (data not shown). Both free and Ch-MCE were activated by Mg^{+2}, Ca^{+2} and Mn^{+2} whereas Hg^{+2} and Co^{+2} inhibited them. Cu^{+2} decrease enzymatic activity of the free MCE by 8.7 % although no lose in activity of Ch-MCE. In general, it was observed that the inhibitory effect of these investigated metal ions was less pronounced with the Ch-MCE compared to free MCE (Pandey et al., 2017). Siar et al. (2017) suggested that if multi-point covalent bond is achieved, the structure of enzyme becomes more rigid and consequently increases enzyme stability under any distorting reagent.

Table 5. kinetic and thermodynamic parameters for casein hydrolysis by free and Ch- MCE.

Parameter	Milk-clotting enzyme	
	Free	Immobilized
E_a (KJ/ mol)	36.76	34.31
V_{max} (U/ mg protein)	10.0	8.0
K_M (mg skim milk/ ml)	28.01	35.71
k_{cat} (S^{-1})	4.3	6.23
k_{cat} / K_M (/S mg/ ml)	0.1535	0.1744
Ka (1/ K_M)	0.036	0.028
ΔH^* (kJ/ mol)	33.86	31.33
ΔG^* (kJ/ mol)	96.68	102.17
ΔS^* (J/ mol /K)	-183.67	-197.87
ΔG^*_{E-T}((kJ/ mol)	5.34	5.12
ΔG^*_{E-S}((kJ/ mol)	9.48	10.5
V_{max} / K_m	0.357	0.224
Q_{10}	1.0	1.0

3.3. Suitability of Ch- MCE in forming milk curds (reusability)

One of the most important characteristics of the immobilized enzymes for both economical and industrial applications is their reusability (El-Shishtawy et al., 2021). The reusability study was carried out under optimal conditions. The Ch-MCE exhibited a very high capability to be reused, since we observed 100 and 90.4 % retained activity after being used for 5 and 7 consecutive cycles, respectively. Conversely, the immobilized proteases retained 40 % of its initial activity after the second cycle (Salazar-Leyva et al., 2017). El-Shishtawy et al. (2021) reported that immobilized enzyme onto CS/ZnO and CS/ZnO/Fe_2O_3 retained 38% and 88% of its original activity after five cycles. Multipoint covalent binding of enzymes on activated supports promotes a rigidification of its structure and reusability (Ahmed et al., 2019). Upon repeated use, gradual decrease in activity was observed (Figure 8), which was probably due to the loss of the enzyme from the carrier physically, frequent interactions between the substrate and the active site of the immobilized enzyme, distorting the active site, and resulting in activity loss (El-Shishtawy et al. (2021)

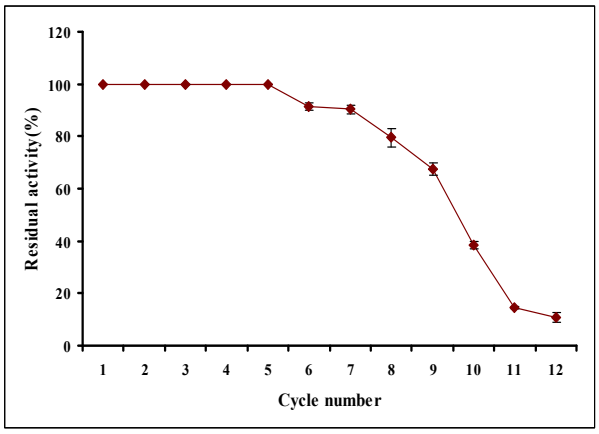

Figure 8. Suitability of Ch-MCE in the forming of milk curds (reusability).

4. Conclusion

Using rennet enzyme in making cheese is one from the largest application of enzymes in food processing. Therefore, this study has aimed to enhance the properties of MCE by immobilization and evaluate its catalytic, kinetics and thermodynamic parameters. MCE from *B. circulans* 25 was covalent-binding to activated chitosan polymer (Ch-MCE) with IY 75% and IE 72%. The Ch-MCE exhibited higher optimum temperature by 10°C and lower activation energy by 0.9- fold than the free form. Additionally, it possessed higher $t_{1/2}$, lower deactivation rate constant, and lower affinity to its substrate. Moreover, the energy for denaturation of the Ch-MCE was 1.8-fold higher than that of the free enzyme, meaning that the immobilization process increases the heat resistance of enzyme. The calculated thermodynamic parameters as enthalpy (ΔH^*), Gibbs free energy (ΔG^*), and entropy (ΔS^*) demonstrated that covalent-binding between enzyme and activated chitosan increased its thermal stability. Furthermore, it was successfully used for 7 consecutive cycles with high retaining activity (90.4 %). It can be concluded that this work helps to overcome the limitations of the reduction in MCE catalytic activity associated with changes in temperature, pH and inhibitors making it useful in industrial applications and biotechnological process.

Acknowledgements

We thank the National Research Centre, Egypt for laboratory assistance.

Competing interests

The authors declare that there is no conflict of interests.

References (please re check References fromat)

Abdella MAA, El-Sherbin GM, El-Shamy AR, Atalla SMM and Ahmed SA. 2020. Statistical optimization of chemical modification of chitosan-magnetic nanoparticles beads to promote *Bacillus subtilis* MK1 α-amylase immobilization and its application. Bull Natl Res Cent. **44**: 1-13.

Abdel-Naby MA, Ismail AMS, Ahmed SA and Abdel-Fattah AF. 1998. Production and immobilization of alkaline protease from *Bacillus mycoides*. Bioresour. Technol. **64**: 205-210.

Afroz QM, Khan KA, Ahmed P and Uprit S. 2015. Enzymes used in dairy industries. Int. j. appl. res. **1(10)**: 523-527.

Ahmed SA, Abdel-Naby MA and Abdel-Fattah AF. 2018. Applicability of wool covalent bonded *Bacillus circulans* 25 cells for milk-clotting enzyme production by batch, repeated batch and continuous process. Braz. J. Chem. Eng. **35**: 847-856.

Ahmed SA, Abdel Wahab WA and Abdel-Hameed SAM. 2019. Comparative study in kinetics and thermodynamic characteristics of immobilized caseinase on novel support from basalt by physical adsorption and covalent binding. Biocatal. Agric. Biotechnol. **18**: 101028.

Cahyaningrum SE and Sianita MM. 2014. Immobilization of pepsin onto chitosan silica nanobeads with glutaraldehyde as crosslink agent. Bull. Chem. React. Eng. Catal. **9**: 263-269. https://doi:10.9767/bcrec.9.3.7060.263-269.

da Silva RR. 2017. Bacterial and fungal proteolytic enzymes: Production, catalysis and potential applications Appl. Biochem. Biotechn. **183**: 1–19.

Dey G, Singh B and Banerjee R. 2003. Immobilization of α-amylase produced by *Bacillus circulans* GRS313. Braz. Arch. Biol. Technol. **46**: 167-176.

Duman YA and Bayer Y. 2021. Kinetics and thermodynamics of keratin degradation by partially purified and encapsulated keratinase from *Bacillus vallismortis* DSM11031. Biocatal. Biotransformati. **39**: 283-291.

El-Shishtawy, RM, Ahmed, NSE and Almulaiky, YQ. 2021. Immobilization of Catalase on Chitosan/ZnO and Chitosan/ZnO/Fe2O3 Nanocomposites: A comparative study. Catalysts. **11**, 820.

Eskandarloo, H. and Abbaspourrad, A. 2018. Production of galacto-oligosaccharides from whey permeate using β-galactosidase immobilized on functionalized glass beads. Food Chemi. **251**:115–124.

Esposito M, Pierro P D, Dejonghe W, Mariniello L and Porta R. 2016. Ezymatic milk clotting activity in artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus*) flowers. Immobilization of alpine thistle aspartic protease, Food Chem. **204**:115–121.

Ferreira MM, Santiago FLB, da Silva NAG, Luiz JHH, Fernandéz-Lafuente R, Mendes AA and Hirata DB. 2018. Different strategies to immobilize lipase from *Geotrichum candidum*: Kinetic and thermodynamic studies. Process Biochem. **67**: 55-63.

Figueira Jde-A, Dias FFG, Sato HH and Fernandes P. 2011. Screening of supports for the immobilization of β-glucosidase. Enzyme Res. Article ID 642460.

Hernandez-Mariinez R, Gutiérrez-Sanchez G, Bergmann CW, Loera-Corral O, Rojo-Dominguez A, Huerta-Ochoa S, Regalado-Gonzalez C and Prado-Barragan LA. 2011. Purification and characterization of a thermodynamic stable serine protease from *Aspergillus fumigatus*. Process Biochem. **46**: 2001–2006.

Hosseini S and Varidi M. 2021. Optimization of Microbial Rennet Encapsulation in Alginate – Chitosan Nanoparticles. Food Chem. **352**:129325.

Lamas EM, Berros RM, Balcão VM and Malcata FX. 2001. Hydrolysis of whey protein by protease extracted from *Cynara cardunculus* and immobilized into highly activated supports. EnzymeMicrob. Technol. **28**: 642-52. .

Lineweaver H and Burke D. 1934. Determination of enzyme dissociation constants. J. Am. Chem. Soc. **56**: 658-666.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the folin phenol reagent. J Biol Chem. **193**: 265-275.

Mafra ACO, Ulrich LG, Kornecki JF, Fernandez-Lafuente R, Tardioli PW and Ribeiro MPdeA. 2019. Combi-CLEAs of glucose oxidase and catalase for conversion of glucose to gluconic acid eliminating the hydrogen peroxide to maintain enzyme activity in a bubble column reactor. J.Catal. **9**: 1-16.

Narwal RK, Bhushan B, Pal A, Malhotra S, Kumar S and Saharan V. 2016. Inactivation thermodynamics and iso-kinetic

profiling for evaluating operational suitability of milk clotting enzyme immobilized in composite polymer matrix. Int. J. Biol. Macromol. **91**: 317-28.

Nwagu TN, Okolo BN and Aoyagi H. 2011. Immobilization of raw starch digesting amylase on silica gel: A comparative study. Afr. J. Biotechnol. **10**: 15989-15997.

Pandey G, Munguambe DM, Tharmavaram M, Rawtani D and Agrawal YK. 2017. Halloysite nanotubes-An efficient nano-support for the immobilization of α-amylase. Appl Clay Sci. **136**: 184–191.

Pervez S, Aman A and Ul Qader SA. 2016. Role of two polysaccharide matrices on activity, stability and recycling efficiency of immobilized fungal amyloglucosidase of GH15 family. Int. J. Biol. Macromol. **96**: 70–77.

Riaz M, Perveen RR, Javed MR, Nadeem H and Rashid MH. 2007. Kinetic and thermodynamic properties of novel glucoamylase from *Humicola* sp. Enzyme Microb Technol. **41**: 558-564.

Salazar-Leyva JA, Lizardi-Mendoza J, Ramirez-Suarez JC, Lugo-Sanchez ME, Valenzuela-Soto EM, Ezquerra-Brauer JM, Castillo-Ya~Nez FJ and Pacheco-Aguilar R. 2017. Catalytic and operational stability of acidic protease from *Monterey sardine* (*Sardinops sagax caerulea*) immobilized on a partially deacetylated chitin support. J. Food Biochem. **41**: e12287._.

Siar E, Zaak H, Kornecki JF, Zidouneb MN, Barbosa O and Fernandez-Lafuente R. 2017. Stabilization of ficin extract by immobilization on glyoxyl agarose. Preliminary characterization of the biocatalyst performance in hydrolysis of proteins. Process Biochem. **58**: 98-104.

Singh RS, Chauhan K and Kennedy JF. 2018. Fructose production from inulin using fungal inulinase immobilized on 3-aminopropyl-triethoxysilane functionalized multiwalled carbon nanotubes. Int. J. Biol. Macromol. **125**: 41–52.

Talbert JN and Goddard JM. 2012. Enzymes on material surfaces. Colloids Surf. B: Biointerfaces. **93**: 8–19.

Thakrar FJ and Singh SP. 2019. Catalytic, thermodynamic and structural properties of an immobilized and highly thermostable alkaline protease from a haloalkaliphilic actinobacteria, *Nocardiopsis alba* TATA-5. Bioresour. Technol. **278**: 150–158.

Wahba MI, Hassan ME and Ali KA. 2021. Chitosan-glutaraldehyde activated carrageenanalginate beads for β-D-galactosidase covalent immobilization. Biocatal. Biotransformation. **39**: 138-151.

Wehaidy HR, Abdel-Naby MA, Shousha WG, Elmallah MIY and Shawky MM. 2018. Improving the catalytic, kinetic and thermodynamic properties of *Bacillus subtilis* KU710517 milk clotting enzyme via conjugation with polyethylene glycol. Int. J. Biol. Macromol. **111**: 296–301.

Yandri, Suhartati T, Satria H, Widyasmara A and Hadi S. 2020. Increasing Stability of α-amylase Obtained from *Bacillus subtilis* ITBCCB148 by Immobilization with Chitosan. Mediterr.J.Chem. **10**: 155-161.

Yang A, Long C, Xia J, Tong P, Cheng Y, Wang Y and Chen H. 2017. Enzymatic characterization of the immobilized alcalase to hydrolyse egg white protein for potential allergenicity reduction. J Sci Food Agric. **97**: 199–206.

Zaboli M, Raissi H, Zaboli M, Farzad F and Torkzadeh-Mahani M. 2019. Stabilization of D-lactate dehydrogenase diagnostic enzyme via immobilization on pristine and carboxyl-functionalized carbon nanotubes, a combined experimental and molecular dynamics simulation study. Arch Biochem and Biophys. **661**: 178–186.