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Comparative study in kinetics and thermodynamic characteristics of immobilized caseinase on novel support from basalt by physical adsorption and covalent binding

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ABSTRACT

Aspergillus niger WA 2017 caseinase was immobilized on a novel support prepared from raw material (basalt) by physical adsorption (PA) and covalent binding (CB). A physicochemical characterization of the more appropriate (glass-ceramic with particle size ≥ 1.0 mm) was performed by means of X-ray diffraction (XRD) and scanning electron microscopy (SEM). One-factor-at-a-time (OFAT) experiments and the response surface methodology (RSM) were used to optimize the immobilization conditions for the maximum immobilization yield (IY %) and to understand the importance and interaction of the affected factors. Under optimized conditions the IY was increased by 2.4 and 1.6-fold, respectively for PA and CB caseinase. Compared to PA caseinase, CB caseinase showed the higher activation energy (*Ea*), half life time ($t_{1/2}$), decimal reduction time (*D*-value), activation energy for denaturation (*Ed*) and Michaelis constant (*Km*). The thermodynamic parameters for irreversible inactivation indicated that enthalpy (ΔH°), Gibbs free energy (ΔG°) and entropy (ΔS°) were higher for CB caseinase than that for PA caseinase within 50–70 °C. Caseinase supported on GC particles by PA and CB retained 21.3 and 57.5%, respectively of its initial activity after 7 consecutive cycles.

1. Introduction

Biocatalysts enhance green processes due to low consumption of chemical and absence of toxic by-products. Proteases have several applications in food, detergent, leather, pharmaceuticals, photographic, biotransformation, biosynthesis and antifouling coating industries (Gohel and Singh, 2013). The application of proteases is further constrained because of their limited stability under extremes conditions. High stability is generally considered an economic advantage because of reduced enzyme turnover.

Enzyme immobilization can involve various supports (organic and inorganic) and various methods (physical adsorption, covalent binding, cross-linking, and entrapment) (Misson et al., 2015). In biocatalysis, there is increasing use of immobilized enzymes due to their advantages such as ease of separation and reuse, improved product quality and purity, increased enzyme (stability, shelf-life, catalytic efficiency) and reduced chances of contamination (Sinha and Khare, 2015; Ricardia et al.,2018). For industrial applications, covalent binding is the most used method where chemical groups on the support surface are activated and react with specifically amino groups on the protein surface of enzyme (Amin et al., 2018). Physical adsorption method of immobilization has the advantages of being inexpensive, chemically simple and enzyme can be physically adsorbed onto particles material. This technique involves the attachment of enzyme to the surface of supports mainly through hydrogen bonding, van der Waals forces, hydrophilic/hydrophobic and electrostatic interactions (Misson et al., 2015; Zdarta et al., 2018).

The catalytic behavior of immobilized enzymes depends on the properties of their supports, such as material types, compositions, and structures (Shojaei et al., 2017). So the selection of the suitable support is a very important part in the immobilization process (Amin et al., 2018). There is no universal carrier that is appropriately suited for all enzymes and all applications. Inorganic materials are notably suitable for industrial purposes due to their excellent properties (mechanical and thermal), biocompatibility, insolubility, non-toxicity and stability over a wide range of temperature, pressure, and pH (Bernal et al., 2014; Jiang et al., 2014; Ahmed et al., 2018; Ricardia et al., 2018).

Modern science and technology always require new materials with special properties to achieve breathtaking innovations. Among all new materials, glass-ceramic (GC) materials play a very special role. GC

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offers the possibility of combining the special properties of conventional sintered ceramics with the distinctive characteristics of glasses (McMillan, 1979). Furthermore, developing GC demonstrates the advantage of combining various remarkable properties in one material (Omar and Abdel-Hameed, 2009).

Almost all the investigated materials used high purity chemical reagents or at least pure processed raw materials such as kaolin, magnetite and quartz sand as the starting batch materials. Due to the high cost of the pure chemicals, and the high melting temperatures or special melting conditions, GC is relatively expensive materials. The GC which produced from raw materials like basalt, metallurgical slag and fly ash are cheaper than those from the elemental technical grade oxide powders. Basalt is a grey to black fine grained volcanic rock, and chemically composed of major oxides of (silicon, aluminum, iron, calcium and magnesium) and of lesser importance oxide of (sodium, potassium, titanium and manganese) as well as trace amounts of other species (El-Shennawi et al., 1999; Ercenk et al., 2012, 2018).

Basalt rocks are molten approximately between 1350 and 1700 °C. When cooled rapidly, basalt solidifies in a glassy amorphous phase (Deák and Czigány, 2009). Basalt glass and GC are used in industrial applications as they are abrasion and corrosion resistant. The production of GC materials from basalt rock is important for the properties inherent in these materials, such as high (resistance, rigidity and chemical stability), low electrical conductivity, good mechanical strength, nearly zero-rated permeability and no bubbles or pores are observed in the structure (Cavdar and Bingol, 2016). Monopyroxenic GC based on basaltic rocks has relatively low melting temperatures and good workability (El-Shennawi et al., 1999).

Immobilization yield (IY %) is affected by many factors such as enzyme concentration, pH, duration time and coupling time. Most of the studies on the optimization of immobilization process changed one separate factor at a time. Response surface methodology (RSM) is recommended for multivariate studies. It is a collection of statistical and mathematical techniques which help to identify the effective factors, interactions between this factors, select optimum conditions and quantify the relationships between measured responses and the vital input factors in limited number of experiments (Eslamipour and Hejazi, 2016; Abdel Wahab and Ahmed, 2018). Central Composite design (CCD) is an effective method for optimization which offers interaction of factors, saving time and effort.

Kinetic and thermodynamic parameters for enzymes change by immobilization, which lead to change of catalytic reaction (Ricardia et al., 2018). Thermodynamic stability involves the resistance to denaturation of a folded protein conformation (Abdel-Naby et al., 2017). Thermodynamic parameters (enthalpy $\Delta H°$, entropy $\Delta S°$ and free energy $\Delta G°$) are good indicators for possibility of using enzyme in biotechnological applications. To the best of our knowledge, studies of *Aspergillus niger* WA 2017 immobilized caseinase (with PA and CB) on GC novel particles (from basalt) have not been reported previously.

The present work mainly focused on employing Egyptian basaltic rocks to prepare nano crystallite diopside solid solution (from pyroxenic family) GC to act as support. Optimizing heat treatment to get nano crystallite diopside ss was studied. Using the produced particles (G and GC) for caseinase enzyme immobilization by CB and PA methods. XRD and SEM were used to determine the developed phases and microstructure. Optimizing immobilization conditions by CCD to enhance immobilization yield (IY %). Additionally, comparative study between PA and CB caseinase on GC catalytic, kinetics and thermodynamics was performed.

2. Material and methods

2.1. Enzyme production

Caseinase enzyme from isolated strain *Aspergillus niger* WA 2017 (GenBank accession number MG873558) has been produced according to the previous work (Abdel Wahab and Ahmed, 2018).

2.2. Preparation of supports

Egyptian basalt (from Abu-Zaabal), limestone (from Samalout), dolomite (from Ataqua), and soda ash were the starting materials for the glass preparations. The chemicals composition was as follows (mol %): 45.54 SiO_2, 2.36 TiO_2, 7.87 Al_2O_3, 4.14 Fe_2O_3, 16.52 MgO and 20.99 CaO (El-Shennawi et al., 1999). The weight percent (%) of natural rock used are: basalt, 75.0–81.3, limestone, 2.7–25.0, dolomite, 15.6–21.1, and soda ash ~3.1. One hundred grams of the batch materials was thoroughly mixed in a mechanical agate mortar for 1 h and then melted in a platinum crucible in an electrically heated Globar furnace. The batches were heated at a rate of ~600 °C/h until melting occurred, at 1400 °C. The melt was maintained at the latter temperature for 0.5 h with occasional agitation to ensure complete homogenization. The melting glass was quenched by pouring them into deionized water.

2.3. Enzyme immobilization

2.3.1. Physical adsorption

Certain weight (0.1 g) of support (glass and glass-ceramic) with different particle size (≤0.35, 0.35–0.7, 0.7–1.0 and ≥ 1.0 mm) was suspended in 0.25 ml of 0.05 M glycine NaOH buffer at pH 10 containing 25 U at 4 °C overnight (Ahmed et al., 2018). The unbound enzymes were removed by washing with the same buffer.

2.3.2. Covalent binding

Support (glass and glass-ceramic) with different particle size (from ≤0.35 to ≥1.0 mm) was activated by glutaraldehyde (GA) as a bifunctional cross linking agent. Activation by 1% GA was carried out by treating particles (0.1 g) with 0.25 ml for 24 h at 4 °C. Subsequently, the activated particles were washed several times with buffer solution. Following the activation, particles were thoroughly washed with sodium acetate buffer to remove the excess GA (Ahmed et al., 2018).

2.4. Characterization of GC particles by SEM and X-ray

Differential Thermal analysis (DTA) of powder glass was performing using 7/Unix system, Perkin–Elmer, Norwalk, CT instrument (El-Shennawi et al., 1999). The structure of the synthesized glass-ceramics was studied by X-ray diffraction (XRD) analysis using Philips X-ray diffractometer PW 1730 with Ni-filtered Cu-K_α X-ray radiation (λ = 1.5406 Å) powered at 40 kV and 30 mA. Diffraction data were recorded in the 2θ range from 10° to 70°, counting for 10 s in steps of $\Delta(2\theta)$ = 0.01°.Scanning electron microscope (SEM) was employed to study the microstructure of the obtained glass-ceramics using J 840A electron prop microanalyzerJeol-Japan.

2.5. Caseinase assay

Enzyme activity was routinely determined by the method of (Daoud et al., 2018) using soluble casein as substrate (Merck, Darmstadt, Germany). Immobilized enzyme on glass and glass-ceramic particles by PA and CB (0.1g) were supplemented to 0.25 ml of casein solution (1.5% in 0.05 M glycine NaOH buffer at pH 10) and were incubated for 30 min at 60 °C. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). The mixture was centrifuged for 20 min at 10,000 × *g* to remove the precipitate. One unit (U) of enzyme activity was defined as the amount of enzyme that librates 1μmole of tyrosine per min under the assay conditions. At least three measurements were made for each experiment and the data given are an average of these results measurements (±) standard deviation. The immobilization yield (IY %) and the residual activity (RA %) were calculated using the following formulae:

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

$$RA\ (\%) = (A_F/\ A_I)\ \times 100 \rightarrow \quad (2)$$

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

2.6. Optimization of immobilization conditions

Immobilization parameters were optimized for maximum IY (%) using one-factor-at-a-time approach for CB immobilization. Then, the Central Composite design (CCD) methodology was used for optimization of other immobilization conditions (for CB and PA methods).

2.6.1. One-factor-at-a-time (OFAT)

In CB process, activation of the glass-ceramic particles (GC) was performed by varying the GA concentrations in the range of 1–11% (v/v). Also, the effect of activation (duration) time was investigated in the range of 3–48 h.

2.6.2. Central Composite design (CCD) to optimize caseinase immobilization

After optimizing the two factors of caseinase immobilization using covalent binding method (GA concentration and duration time), Central Composite design was implemented to optimize the other factors for immobilization process either using covalent binding (CB) or physical adsorption (PA) methods. Two variables (enzyme concentration and coupling time) were chosen for response surface methodology of CCD. Table 1 shows CCD (with coded and real values) for CB and PA immobilization methods respectively. The following second order polynomial equation using a multiple regression analysis was used to express CCD results

$$Y = \beta_0 + \Sigma\ \beta_i X_i + \Sigma\ \beta_{ii} X_i^2 + \Sigma\ \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the interactive coefficients, and x_i and x_j the coded independent variables.

Table 1
CCD matrix (with coded and real values) for caseinase immobilization on nano particle GC by covalently binding (CB) and physical adsorption (PA) methods.

Run	Factor 1	Factor 2	Covalently binding		Physical adsorption	
	a [enzyme conc.] [U/ml]	b [coupling time] [h]	IY(%) [R1]	Predicted value	IY(%) [R2]	Predicted value
1	767.5 (0)	17 (0)	58.88	50.82	63.55	62.85
2	767.5 (0)	17 (0)	44.66	50.82	63.17	62.85
3	767.5 (0)	17 (0)	48.39	50.82	63.03	62.85
4	1287.2 (+2)	17 (0)	85.68	76.00	43.25	42.64
5	1287.2 (+2)	17 (0)	85.93	76.00	43.86	42.64
6	767.5 (0)	17 (0)	58.02	50.82	63.66	62.85
7	767.5 (0)	17 (0)	58.21	50.82	63.12	62.85
8	767.5 (0)	17 (0)	58.42	50.82	63.12	62.85
9	400.0 (−1)	28 (+1)	54.74	55.54	72.05	71.51
10	400.0 (−1)	6 (−1)	75.21	77.54	73.54	72.81
11	1135.0 (+1)	6 (−1)	50.14	58.31	32.26	32.75
12	1135.0 (+1)	28 (+1)	38.67	45.31	32.38	33.06
13	247.7 (−2)	17 (0)	97.70	96.84	97.20	98.15
14	247.7 (−2)	17 (0)	97.30	96.84	98.22	98.15
15	767.5 (0)	17 (0)	54.89	50.82	63.51	62.85
16	767.5 (0)	17 (0)	50.30	50.82	63.63	62.85
17	767.5 (0)	17 (0)	50.71	50.82	63.89	62.85
18	767.5 (0)	1.4 (−2)	49.85	44.30	34.86	35.02
19	767.5 (0)	32.5 (+2)	22.96	19.56	34.42	34.31

2.6.2.1. Validation of the model.

Validity of the model was checked through two experimental combinations conducted according to the conditions predicted by the model. The results were compared with the predicted values.

2.6.2.2. Statistical analysis.

The model was statistically analyzed using the analysis of variance (ANOVA).

2.7. Characterization of immobilized enzyme

2.7.1. Effect of temperature on caseinase activity

Effect of temperature on caseinase activity was studied by measuring the activity of the immobilized enzyme (PA and CB) at different temperature varying from 30 to 70 °C. The relative activities were determined. The activation energy (*Ea*) was calculated from the slope of the Arrhenius plot according to the following equation:

$$Slope = -\ Ea\ /\ (2.303RT) \rightarrow \quad (3)$$

Where: R is the gas constant (8.314 kJ/mol) and T is the absolute temperature (Kelvin).

2.7.2. Effect of the reaction time on caseinase activity

The enzyme assay (PA and CB caseinase) was conducted at 60 °C and pH 10 for different time intervals (10–60 min).

2.7.3. Effect of pH on caseinase activity

Optimal pH for PA and CB caseinase activity was determined according to Daoud et al. (2018) by measuring the activity at different pH values (7- 12) in 0.05 M of the appropriate buffer. The relative activities were determined.

2.7.4. Effect of substrate concentration

Caseinase was assayed as described before and the assay contained with varying amounts of casein (0.25–3.0%). The enzyme kinetic parameters, Michaelis–Menten constant (*Km*) and maximum reaction velocity (*Vmax*) were calculated using the Lineweaver–Burk plot (Lineweaver and Burk, 1934) at optimum assay conditions.

2.7.5. Thermal stability and thermodynamic properties of caseinase enzyme

The thermal inactivation of immobilized enzyme (PA and CB) was evaluated by incubating the enzyme at temperatures 50, 60 and 70 °C without substrate (Daoud et al., 2018). After defined time intervals (15, 30, 45 and 60 min), the samples were cooled to stop thermal inactivation and the residual activities were determined. All the thermodynamics were calculated with the following equations (4-10) as reported by (Abdel-Naby et al., 2017; Mostafa et al., 2018). The deactivation rate constant (*kd*) was calculated when log of residual activity (%) was plotted against time (min) at the temperatures used for inactivation studies.

$$kd = \text{-Slope} \rightarrow \quad (4)$$

Decimal reduction time (*D*-value) was defined as the time required to reduce 90% of the initial activity at the used temperature. The *D*-value and the half-lives ($t_{1/2}$) of the immobilized enzyme were determined from the relationships

$$D\text{-value} = \ln 10/\ kd \rightarrow \quad (5)$$

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

The activation energy for caseinase denaturation (*Ed*) was determined by a plot of log denaturation rate constants (ln *kd*) versus reciprocal of the absolute temperature (*K*) using the following equation:

$$Slope = -Ed/\ R \rightarrow \quad (7)$$

The change in enthalpy (ΔH°, kJ/mol), free energy (ΔG°, kJ/mol) and entropy (ΔS°, J/mol/K) for thermal denaturation of caseinase were determined using the following equation:

$$\Delta H^\circ = Ed - RT \rightarrow \quad (8)$$

$$\Delta G^\circ = -RT \ln (Kd.h/\ Kb.T) \rightarrow \quad (9)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/\ T \rightarrow \quad (10)$$

Where: *Ed* is the activation energy for denaturation (KJ/mol)), R is the gas constant (8.314 J/mol/K), T is the corresponding absolute temperature (K), *kd* is the deactivation rate constant (/min), h is the Planck constant (11.04×10^{-36} J min) and *Kb* is the Boltzman constant (1.38×10^{-23} J/K).

2.7.6. Stability in presence of some additives and inhibitors

Immobilized caseinase (PA and CB) was pre-incubated at 30 °C for 1 h with various detergent additives and inhibitors (H_2O_2, SDS, tween 40, triton X-100, urea and EDTA) with different concentrations (Daoud et al., 2018). The reaction without additive was taken as control (100%).

2.7.7. Operational stability (Reusability)

The operational stability (reusability) of the immobilized caseinase (PA and CB) was assessed by repeated hydrolysis reaction of substrate (casein) in the buffer system over several consecutive cycles. At the end of each cycle, the immobilized caseinase was separated by filtration, was washed three times with the same buffer and was used for new cycle. The activity of the immobilized enzyme was considered to be 100% in the initial cycle. Activity in each cycle was defined as the residual activity of the immobilized caseinase.

3. Results and discussion

3.1. Immobilization of caseinase by covalent binding and physical adsorption

The results in Fig. 1 showed that, GC is more suitable support than G and the larger particle sizes are more convenient than the smaller one. The highest IY % (59.7 and 40.91%) was obtained by CB and PA of caseinase to GC with particle size ≥ 1.0 mm consequently, they were used for the following experiments. The structural studies of glassy phase (G) revealed short range order (SRO) while crystalline phase or GC is characterized by long rang ordering (LRO). Consequently, in the case of glass, no microstructure is evident. In GC, microstructure plays a very important role in the properties of the material, where presence of high amount of crystals cases increase in the surface area. On addition, the random edges and corners of the crystals, especially in the GC, allow more attachment for enzymes on it contrary to amorphous or glassy phase which have no microstructure and have smooth surface. Physical adsorption yielded lower IY % than CB possibly due to excess leaching of the enzyme due to weaker binding forces. In addition, reduction in enzyme activity upon PA immobilization might be due to block the active site of enzyme by the immobilization agent (Madhavan et al., 2017). Misson et al. (2015) reported that multiple point covalent bonding via short spacer arms on carriers may be a powerful strategy to improve enzyme activity. Moreover, increase the distance between carrier and the enzyme molecule leads to an increase the quantity of bonded protein and then to increase the activity of immobilized enzyme.

3.2. Optimization of immobilization conditions

Immobilization conditions were optimized for maximum immobilization yield (Sinha and Khare, 2015).

3.2.1. One-factor-at-a-time

The effect of glutaraldehyde (GA) concentration as cross linker for support activation was presented in Fig. 2. At 3% GA concentration, the immobilization yield for CB was enhanced by 1.4-fold. The results indicate a gradual loss in enzyme activity at higher concentrations of GA. Glutaraldehyde, a apart from being a cross linker can also acts as a denaturing agent (Sinha and Khare, 2015). At the same time, higher GA concentration increased the amount of bound enzyme to the support causing change in the spatial structure of the enzyme active center and consequently decreases the activity (Ahmed et al., 2018). At low GA concentrations (less than 3%), the activity of immobilized enzyme was

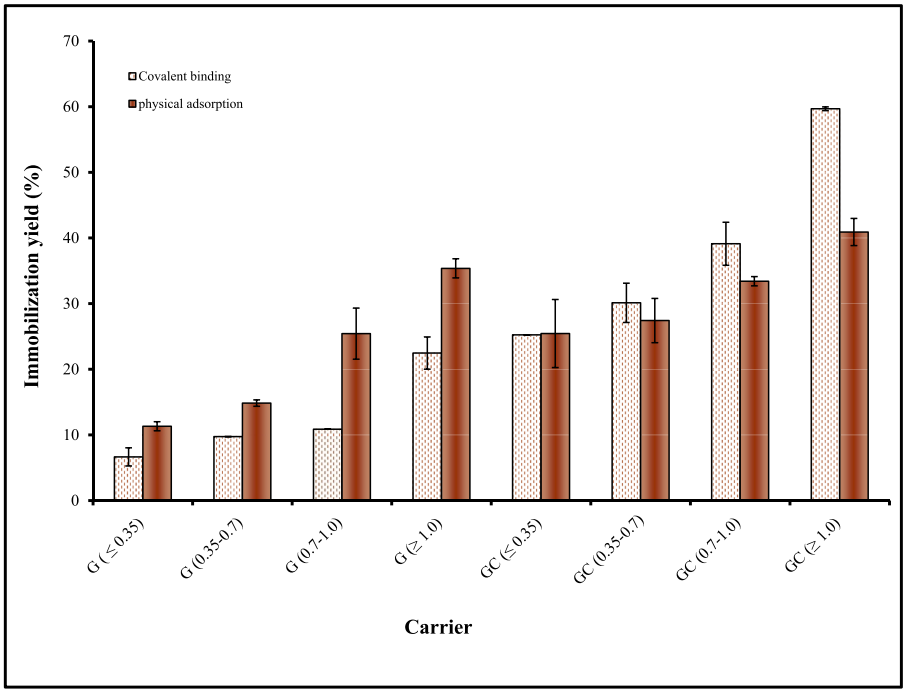

Fig. 1. Immobilization of caseinase enzyme on glass and glass-ceramic with different particle sizes by covalent binding and physical adsorption.

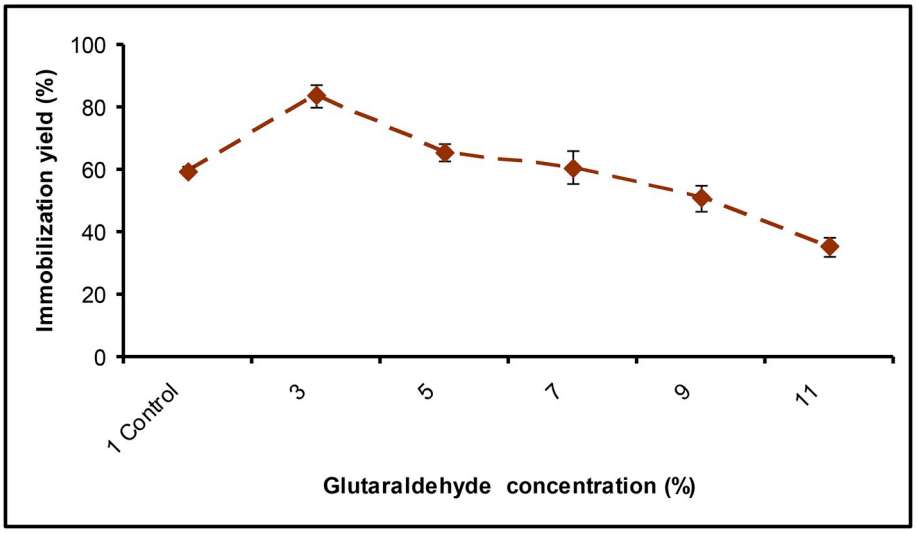

Fig. 2. Effect of glutaraldehyde concentration on immobilization yield of caseinase. on GC (≥1.0) by covalent binding.

Table 2
Coefficients in Terms of Coded Factors for CCD design of CB and PA caseinase.

Source	Coefficient Estimate		Standard Error		VIF	
	CB	PA	CB	PA	CB	PA
Intercept	50.82	62.85	2.19	0.2993		
Whole-plot Terms:						
a-enzyme conc.	−7.37	−19.63	1.83	0.2528	1.00	1.00
a^2	17.80	3.77	1.83	0.2520	1.01	1.01
b^2	−9.45	−14.09	2.18	0.3015	1.01	1.01
Subplot Terms:						
b-coupling time	−8.75	−0.2491	2.06	0.2935	1.00	1.00
ab	2.25	0.4031	2.92	0.4151	1.00	1.00

lower possibly due to insufficient cross linking, leading to leach of enzyme and decrease IY (Torabizadeh et al., 2014). On the other side, change in duration time did not affect the immobilization yield (IY %) for CB caseinase (data not shown). Longer activation time (> 24 h), excessive cross-linking restricts the enzyme's flexibility, and limits mass transfer consequently, reduced enzymatic activity (Torabizadeh et al., 2014).

3.2.2. Central Composite design (CCD) for optimizing caseinase immobilization

The most significant parameters affect the immobilization process (enzyme concentration and coupling time) were undergone the CCD statistical analysis in order to determine the optimal concentration of each variable. Table 1 shows the design form and the corresponding experimental data of the two independent variables for CB and PA immobilization methods respectively. The coefficients and P-values of the variables of linear (a, b), quadratic (a^2, b^2) and interactions (ab), terms were determined for both CB and PA immobilization methods, respectively (Table 2). P-values less than 0.05 indicate model terms are significant. In the covalently bonded method, a, B, (linear coefficients), a^2, B^2 (quadratic coefficients) are significant model terms optimizing the immobilization process. However, the interaction of the two terms, (aB) was not significant factor scoring P-value of 0.4671. It is well known that, P-Values greater than 0.10 indicate the model terms are not significant. It is worthy to mention that the success in optimizing the immobilization process is measured by the increase scored in the immobilization yield IY %. The value of R^2 in the model is usually an evident to its validity and fitness, in the present model, R^2, was 0.9314, indicating that the model can explain about 93.14% of the variability in the response. Also, the closure to the adjusted R^2value (0.8878) reflex validity of the model. F-test analysis of variance (Table 3) was used to determine the statistical significance of the second-order model equation. The model F-value of 46.59 implies the model is significant. There is only a 0.54% chance that an F-value this large could occur due to noise. Concerning the PA method, only the enzyme concentration (a) from the linear coefficients, and (a^2, B^2) from the quadratic coefficients are significant model terms, scoring P-values less than 0.05. Values greater than 0.10 indicate the model terms are not significant. The model (Table 3) explained about 99.86 of the variability in the immobilization yield results as indicated from R^2 value (0.9986). One of the most important factors which ensure the model validity is the closure between R^2 and Adjusted R^2 values (0.9977). Also the model scored very low C.V. % value (1.44%). All results, the indicate validity of the design. The significance of the model is also clear from the F-value (2867.13), there is only a 0.01% chance that an F-value this large could occur due to noise.

Table 3
ANOVA for CCD of caseinase immobilization on GC nanoparticle by CB and PA.

Source	Term [df]		Error [df]		F-value		p-value		CB PA
	CB	PA	CB	PA	CB	PA	CB	PA	
Whole-plot	3	3	2.97	4.51	46.59	2867.13	0.005	< 0.0001	significant
a-enzyme conc.	1	1	3.52	5.27	16.27	6026.20	0.020	< 0.0001	
a^2	1	1	2.58	3.99	94.89	224.05	0.004	0.0001	
b^2	1	1	2.82	4.33	18.72	2185.26	0.026	< 0.0001	
Subplot	2	2	6.77	8.54	9.27	0.832	0.012	0.468	significant
b-coupling time	1	1	6.77	8.54	17.95	0.720	0.004	0.419	
ab	1	1	6.77	8.54	0.594	0.943	0.467	0.358	

CB: R^2 = 0.9314, Adjusted R^2 = 0.8878, C.V. % = 10.48. PA: R^2 = 0.9986, Adjusted R^2 = 0.9977, C·V. % = 1.44.

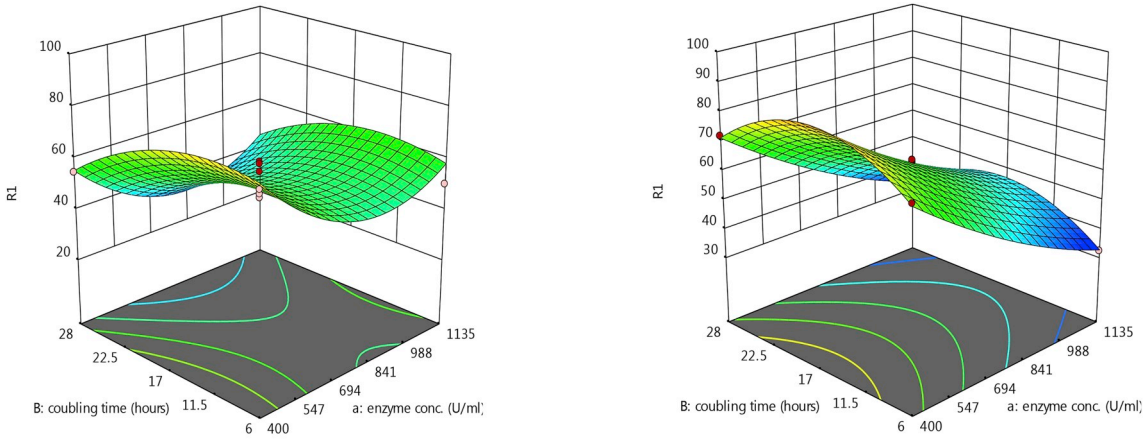

Fig. 3. Three dimensional (3D) response surface graph of caseinase immobilization using covalent binding (A) and physical adsorption (B) methods showing the interaction between the two variables: (a) enzyme concentration and (B) coupling time.

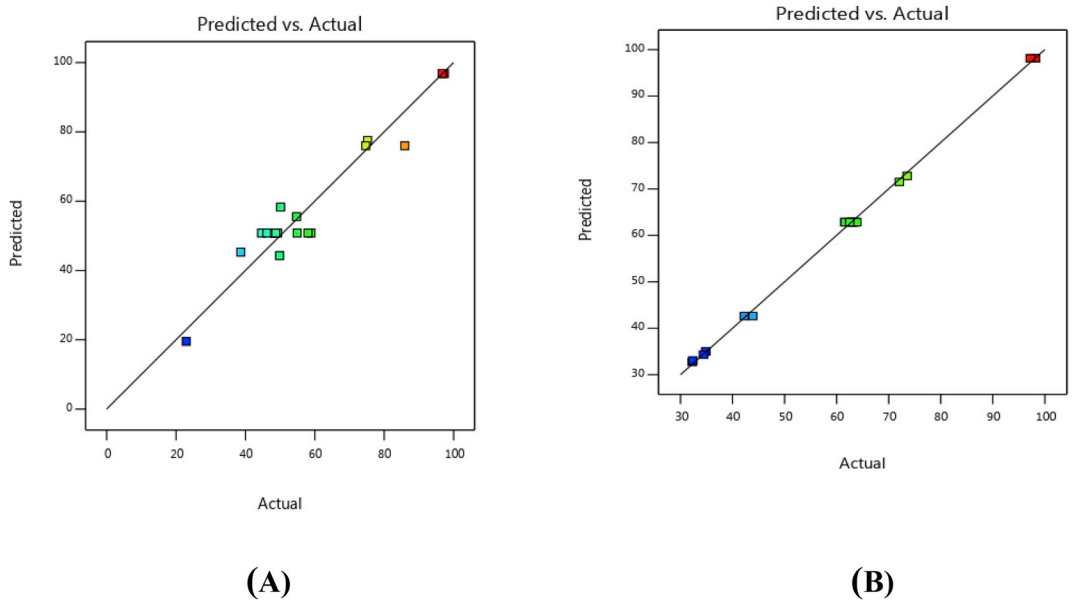

Fig. 4. The relation between predicted and actual values for caseinase immobilization using covalent binding (A) and physical adsorption (B) techniques using CCD.

The final optimized conditions for immobilization were 247.78 U/ml enzyme conc. for 17 h coupling time for both tested immobilization methods (CB and PA) scoring IY (%) 97.33, 98.22%, respectively. The optimization process for coupling protease with the soluble–insoluble polymer (Eudragit S-100) using full factorial design lead to 45% activity yield (Silva et al., 2006). Maximum immobilization yield (63.5% and 79.77%) was recorded for protease immobilization into the amorphous mesoporous silica (SBA-15) and crystalline mesoporous zeolite (Nano-ZSM-5), respectively using entrapment method (Kumari et al., 2015). Chae et al. (1998) reported that the optimum enzyme concentration for protease immobilization using LewatitR258K activated with GA was 1.8 mg/ml which was very lower than our result (6.0 mg/ml). The relationships between the immobilization yield (response) and experimental levels of each variable for both CB and PA immobilization methods were illustrated using three dimensional (3D) response surface graphs Fig. 3 (A and B) respectively.

In the present study, the optimization process of caseinase immobilization was undergone the complementary effect of two effective strategies which are one-factor-at-a-time for optimizing GA concentration and time for duration followed by CCD for enzyme concentration and coupling time. The model validity for both methods (CB and PA) was clear when comparing the experimental value of caseinase IY (97.33 and 98.22%) with the statistical predicted value (96.84 and 98.15%), which are closely related. Fig. 4 (A and B) shows the close relation between actual and predicted values through the design for both methods (CB and PA) respectively. The optimization process showed great effect on the caseinase immobilization, the IY of caseinase using CB method scored 97.33% with about 1.6 times the original yield (59.9%), while for the physical adsorption, the progress in the immobilization yield 98.22% scored about 2.4 time higher than the original value (41.86%). Fig. 5 (A and B) shows Box-Cox plot for power transformation of caseinase immobilization using CB (A) and PA (B) methods. The confidence level (CL) for Lambda using CB was (−0.28, 1.58), the best Lambda value is 0.54. While for PA, (CL) for Lambda was (0.97, 1.33) scoring (1.15) as the best value.

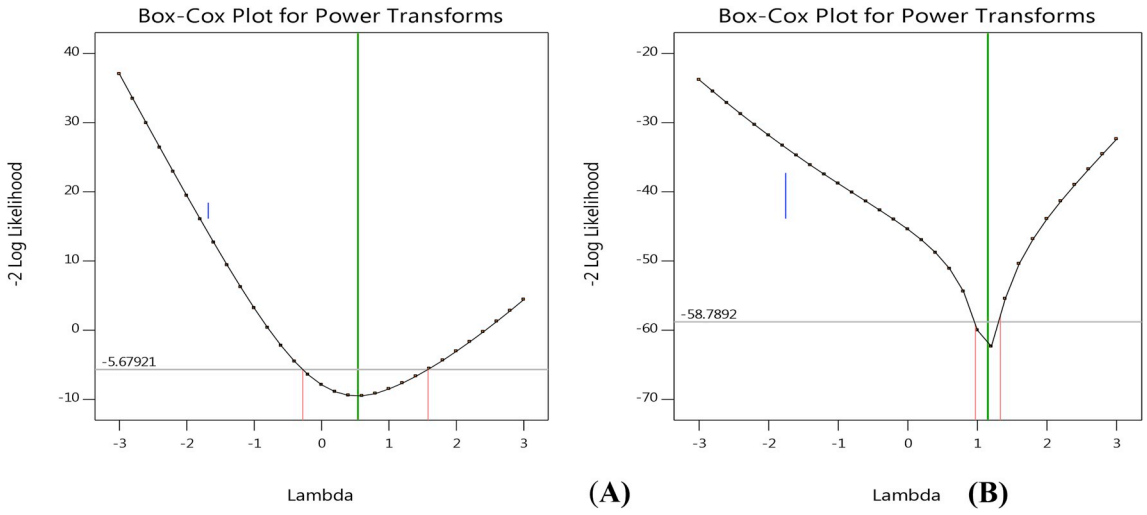

Fig. 5. Box-Cox plot for power transforms of caseinase immobilization using covalent binding (A) and physical adsorption (B) techniques using Central Composite Design.

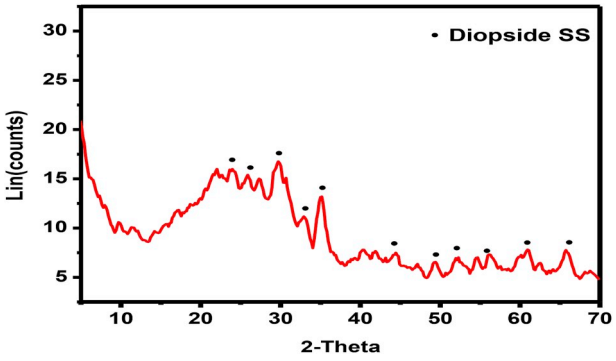

Fig. 6. XRD of glass-ceramic base on Egyptian basaltic rocks, revealing crystallization. of diopside solid solution (ss).

3.3. Characterization of glass-ceramic particles by X-ray and SEM

3.3.1. X-ray diffraction analysis

In the previous work (El-Shennawi et al., 1999), different heat treatments were applied using one stage programs; however none of these programs produce nano size crystals immersed in glass phase. Consequently, suggesting heat treatment on two steps, one at the nucleation temperature for long time (675 °C/10 h) which can produce a large amount of nuclei (pre crystallization step) followed by another one at the crystallization temperature (890 °C/2 h) to get the crystal growth, was applied. Presence of large number of nuclei seems to prevent the crystal from more growth, so nano crystallite of pyroxenic structure structure distributed in glassy matrix, expected to be obtained, as seen later. Fig. 6 depicts XRD for the prepared glass ceramic after heat treatment at 675 °C/10 h + 890 °C/2 h. The XRD patterns are well matched with the Joint Committee on Powder Diffraction Standards (JCPDS) card No. 75–1092 revealing crystallization of pure diopside solid solution (ss) in its monoclinic form; the noisy background revealed presence of amorphous phase. The broadness of Bragg's peak revealed crystallization of nano size crystals. The average crystallite sizes were calculated from the most intense XRD peaks using Debye-Scherrer formula:

$$D = k\lambda/B \cos\Theta$$

Where D is the particle size, k is constant, λ for Cu is 1.54 Å, B is the full half wide and $2\Theta = 4^{\circ}$. The crystallite sizes locate in the nanorange where it recorded < 50 nm.

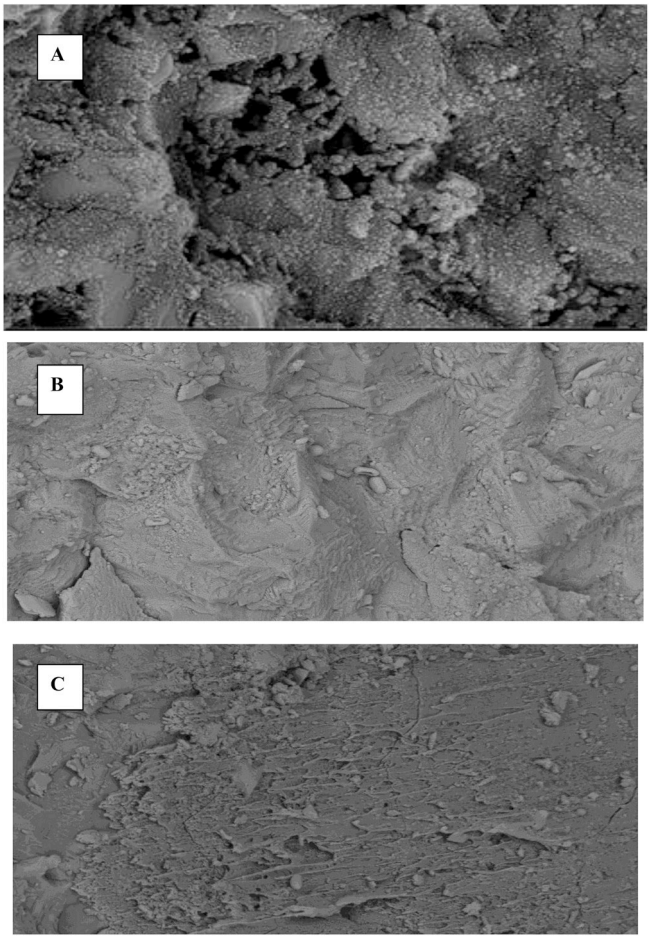

Fig. 7. SEM of mono pyroxenic glass-ceramic based on Egyptian basaltic rocks (A) native, (B) immobilized caseinase by physical adsorption and (C) immobilized caseinase by covalent binding.

3.3.2. Scanning electron microscope (SEM)

Scanning electron microscope (SEM) is a tools used to observe the morphology of a sample at higher magnification and higher resolution. Depending upon the extent of ordering of the atoms or molecules, a material may be classified as crystalline or glassy. The structural studies of glassy phase revealed short range order (SRO) while crystalline phase

or glass ceramic is characterized by long rang ordering (LRO). Consequently, in the case of glasses, no microstructure is evident. However, in glass-ceramics, microstructure plays a very important role in the properties of the material, where presence of high amount of crystals cases increase in the surface area. On addition, the random edges and corners of the crystals, especially in the nano range, allow more attachment for enzymes on it contrary to amorphous or glassy phase which have no microstructure and have smooth surface. In this study, SEM (Fig. 7) shows microstructure for the GC support before enzyme immobilization (Fig. 7A) and after immobilization via physical and covalent methods, (Fig. 7B and C, respectively). Native GC (before immobilization) revealed massive crystallization of nano size diopside solid solution crystals in rounded form. Presence of one kind crystal form certifies the results obtained from XRD where pure pyroxenic phase was developed. Crystallization of nano size GC revealed the success of heat treatment schedule applied. Fig. 7B for GC immobilized by PA method depicts blinded texture where the crystals boundaries are not clear, as if it were covered by enzyme molecules. Using CB method (Fig. 7C) revealed flow of caseinase enzyme on the surface of the basaltic GC carrier as sea waves cased by covering the surface with caseinase enzyme, so the crystal structure was totally blended. Similarly, Nasir et al. (2017) attributed the change in morphology of composites in SEM structural analysis to their interaction.

3.4. Characterization of CB and PA caseinase

The immobilized enzyme properties depend on the nature of the support and the method used for immobilization. Change may be induced via some molecules presented in the surfaces of support. Changes in enzyme structure tend to decrease enzyme activity (Sinha and Khare, 2015). Such enzyme-solid interactions also play a major role in the denaturation, stability, refolding, and degradation of the bound enzyme (Shojaei et al., 2017).

3.4.1. Effect of temperature on caseinase activity

A comparative study between PA and CB caseinase was performed at different temperature and the relative activity was shown in Fig. 8A. The results revealed that the optimum temperature for both PA and CB caseinase was 60 °C. At higher temperature (70 °C) lose in activity of CB and PA caseinase was 10 and 17%, respectively. Stability at higher temperature of CB caseinase could be the result of an improvement in enzyme rigidity through covalent immobilization. Proteases are widely used in many processes especially in detergent industry which is the largest market for enzymes at 25–30% of total sales. In fact, a detergent enzyme must withstand many harsh conditions such as surfactants, oxidants, and variation of temperatures up to 60 °C (Daoud et al., 2018). Activation energy (*Ea*) calculated for CB caseinase was 36.19 kJ/mol, which was higher than that of PA caseinase (4.40 kJ/mol) by 8.4 times. Similar observation was obtained by Puentes-Camacho et al. (2017) on immobilized lysozyme by covalent binding which was higher than that obtained by physical adsorption. The result suggested that immobilization by PA improved the quality of caseinase by lowering down the energy required to make the activated complex of enzyme-substrate (Abdel-Naby et al., 2017).

3.4.2. Effect of the reaction time on caseinase activity

The results presented in Fig. 8B revealed that 10 and 15 min are the suitable time for maximum activity of CB and PA caseinase, respectively. Moreover, the relative activity of CB caseinase was higher than that of PA caseinase by 1.4 times. Reaction times higher or lower than the optimum values did not affect the activity and consequently IY%. Mittal et al. (2005) reported that 90 min is the optimum time for maximum activity of immobilized protease.

3.4.3. Effect of pH on caseinase activity

The highest activity was obtained at optimum pH (10) for both CB and PA caseinase. According to the results (Fig. 8C), pH variations have less effect on the PA caseinase than CB caseinase. Whereas at pH 11 the relative activity decreased by 15 and 1.64% of its activity for CB and PA, respectively. Proteases are widely used in detergent industry which is the largest single market for enzymes at 25–30% of total sales. Daoud et al. (2018) reported that a detergent enzyme must withstand to harsh conditions such as high pH values (8.0–10.5) and variation of temperatures up to 60 °C. The results indicated the possibility of using the immobilized enzyme as bio-additive in the detergent.

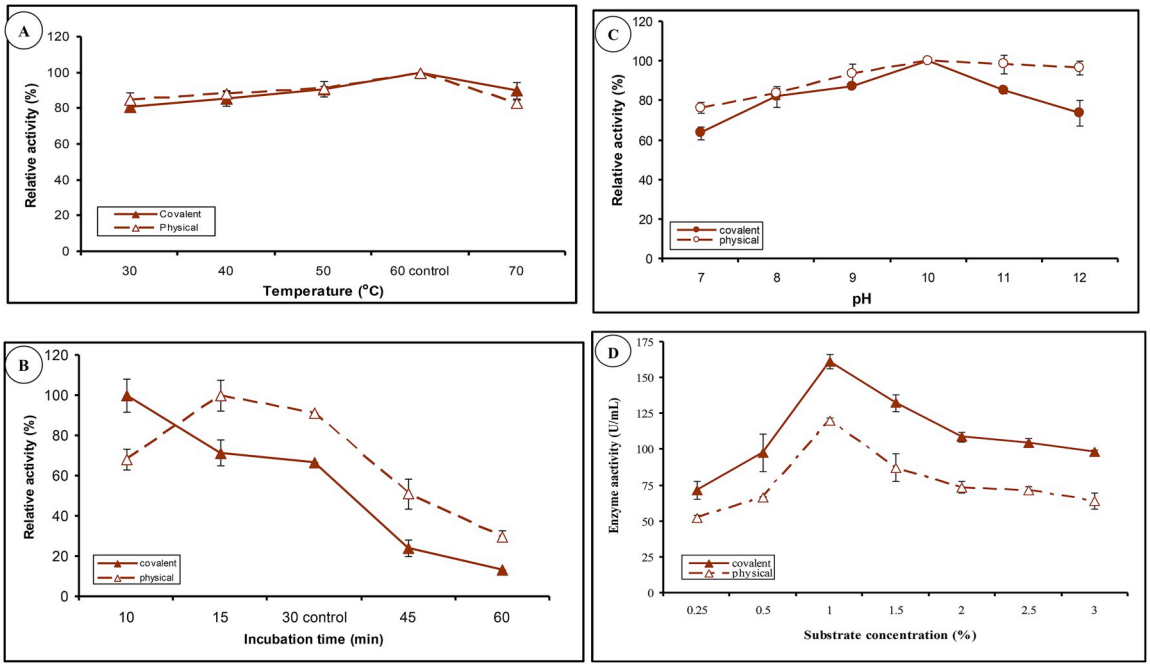

Fig. 8. Effect of temperature (A), incubation time (B), pH (C) and substrate concentration (D) on the activity of immobilized caseinase by covalent binding and physical adsorption.

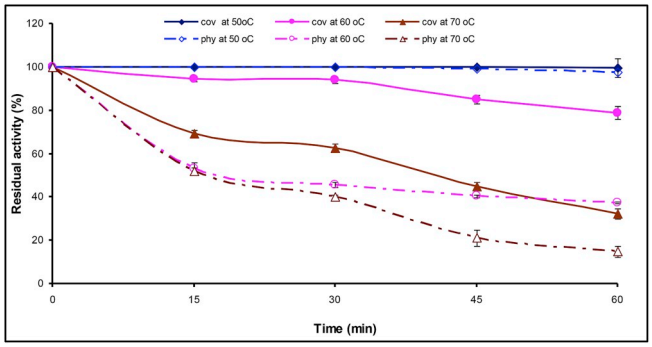

Fig. 9. Thermal stability of immobilized caseinase by covalent binding and physical adsorption.

3.4.4. Effect of substrate concentration

The highest activity for CB and PA caseinase was obtained at 1% casein concentration (Fig. 8D). In addition, activity of CB caseinase was higher than that of PA caseinase by 1.4 fold at optimum concentration. The kinetic parameters of the CB and PA caseinase were calculated using casein as substrates (data not shown). The 1.2 times higher *Km* of CB caseinase (1.69 mg casein/ml) than PA caseinase (1.47 mg casein/ml) implies a decreased affinity between enzyme active site and substrate. This effect might be a consequence of changes of enzyme configuration after immobilization. The change in rigidification of the enzyme molecule depends on the immobilization methods. The *Km* value gives an idea about the affinity of an enzyme to its substrate (Sinha and Khare, 2015). Decreased *Km* of immobilized enzyme by PA indicated the increased affinity between substrate and enzyme active site and consequently, enzyme activity. This may be caused by restricted mobility of the covalent bound enzyme and reduced accessibility of the substrate to the active site (Sinha and Khare, 2015). On the other side, calculated *Vmax* for PA and CB caseinase was the same value 100 U/mg protein. The *Vmax/Km* ratio of PA caseinase (68.03 U/mg protein/mg casein/ml) which was 1.2- time higher than that of CB caseinase (59.17 U/mg protein/mg casein/ml).

3.4.5. Stability to heat inactivation

3.4.5.1. Thermal properties. Enzyme stability is an important key to increase its economic value and managing their application. So, the stability of PA and CB caseinase at different temperatures was studied to elucidate differences between the methods of immobilization. As shown in Fig. 9, thermal stability of the caseinase was significantly higher upon immobilization by CB than that of PA. At 60 °C, PA caseinase lost 59.7% of its initial activity while the CB caseinase preparation retained 85% of its activity after 45 min. The result showed that after 30 min at 70 °C the CB caseinase kept 62.7% of its activity, while the PA caseinase lost 59.9% of its initial activity. Thermal properties of the CB caseinase and PA caseinase were provided in Table 4. The deactivation rate constant (*kd*) for PA caseinase is higher than that of CB caseinase at 50, 60 and 70 °C. For example, the *kd* at 70 °C for the PA and CB caseinase were 13.68×10^{-3} and 7.8×10^{-3}/min, respectively. Multipoint covalent attachment of enzymes on activated supports promotes a rigidification of its structure consequently, this rigidification reduces any conformational change involved in enzyme inactivation and increases its stability (Pal and Khanum, 2011). Moreover, activation energy of denaturation (*Ed*) of CB and PA caseinase were 257.17 and 209.18 kJ/mol, respectively. The higher value of *Ed* indicates that more energy is required to enzyme denaturation (deactivate the complex linked covalently) and hence implied to be more thermostable (Puentes-Camacho et al., 2017). In addition, the calculated $t_{1/2}$ and *D*-values values at 50, 60 and 70 °C of the CB caseinase were higher than that of PA caseinase. The higher stability of CB enzyme may be attributed to the multipoint covalent binding of the enzyme molecules to the support which restricts the flexibility and conformational mobility of the enzyme, thus preventing unfolding or denaturation upon heating (Sinha and Khare, 2015). The covalent binding method also increased $t_{1/2}$ and thermal stability of enzymes. The conferred stability of covalent bond enzyme comes from unlimited covalent binding between the substrate and the enzyme due to the lack of any barrier between them (Mohamad et al., 2015).

3.4.5.2. Thermodynamic parameters. The thermodynamic parameters of substrate hydrolysis by the CB and PA caseinase was presented in Table 4. The activation enthalpy of denaturation (ΔH°) is important thermodynamic parameter expressing the require amount of energy to denature the enzyme. Therefore, large and positive values of ΔH° should be associated with high enzyme thermostability. At 50 °C ΔH° was 254.48 and 206.49 kJ/mol, respectively for CB and PA caseinase and the values were remained the same up to 70 °C. Similar results have been documented for immobilized pullulanase by covalent binding method (Singh et al., 2010). Also, ΔH° of CB caseinase was ~1.2 times higher than that of PA caseinase at different temperatures. Higher ΔH° suggested an increase of the stability of the enzymatic system assuming that during inactivation there are fewer bonds broken (Azevedo et al., 2015). Similar observation was obtained by Puentes-Camacho et al. (2017). Covalent binding immobilization led to conformational change, thus the enzyme became more rigid and thermally stable. The Gibbs free energy (ΔG°) for activation of thermal unfolding of immobilized caseinase by CB and PA were 107.29 kJ/mol and 102.96 kJ/mol, respectively at 50 °C. With an increase in temperature, a decrease in ΔG° was observed. ΔG° is a more accurate and reliable predicting tool to evaluate enzyme stability because it includes both enthalpic and entropic contributions. A negative or smaller value of such (ΔG°) is associated with a more spontaneous process (decreased thermostability) and more easily undergoes denaturation (Souza et al., 2015). The ΔG° of the PA caseinase is lower than that of CB caseinase suggested that the conversion of a transition state of enzyme-substrate complex into a product was more spontaneous as reported by Riaz et al. (2007). The free energy of Gibbs (ΔG°) of activation was positive for both form (CB and PA) and the inactivation was not spontaneous Puentes-Camacho et al. (2017). The activation entropy (ΔS°) is the amount of energy for each degree involved in the transition from original to denatured state (Souza et al., 2015). As shown in Table 4, the ΔS° values were positive at all temperatures and the ΔS° for PA caseinase were lower than that of CB caseinase. Puentes-Camacho

Table 4
Kinetic and thermodynamic parameters for thermal inactivation of CB and PA caseinase.

Temperature (°C)	k_d (/min)		$t_{1/2}$ (h)		*D*-value (h)		ΔH° (kJ/mol)		ΔG° (kJ/mol)		ΔS° (J/mol/K)	
	CB	PA	CB	PA	CB	PA	CB	PA	CB	PA	CB	PA
50	0.03×10^{-3}	0.15×10^{-3}	379.09	75.36	1259.33	250.33	254.48	206.49	107.29	102.96	0.46	0.23
60	1.69×10^{-3}	6.49×10^{-3}	6.85	1.78	22.75	5.91	254.40	206.41	99.53	96.64	0.47	0.33
70	7.8×10^{-3}	13.68×10^{-3}	1.48	0.84	4.92	2.81	254.32	206.33	98.24	95.81	0.46	0.32

k_d = thermal inactivation rate constant, $t_{1/2}$ = half-life, *D*-value = decimal reduction time, ΔH° = variations in enthalpy; ΔG° = variations in free energy; ΔS° = variations in entropy.

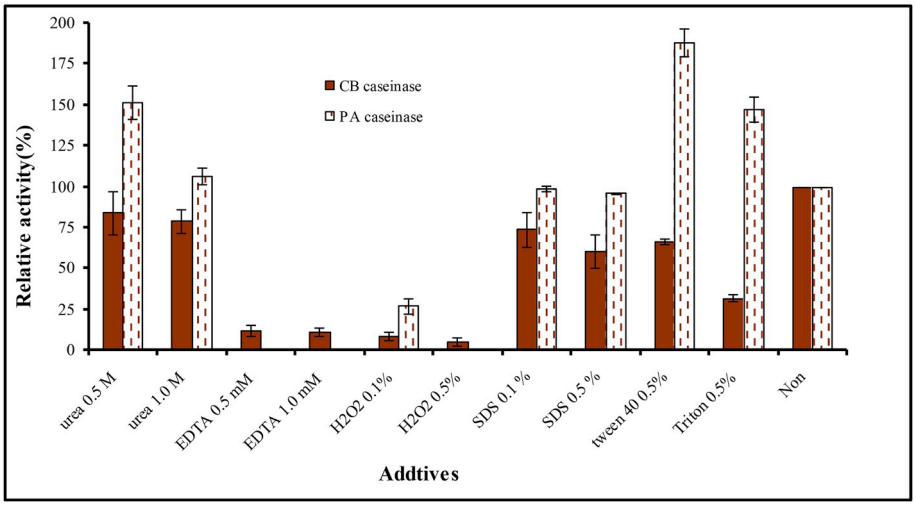

Fig. 10. Effect of some additives and inhibitors on covalent binding and physical adsorption caseinase.

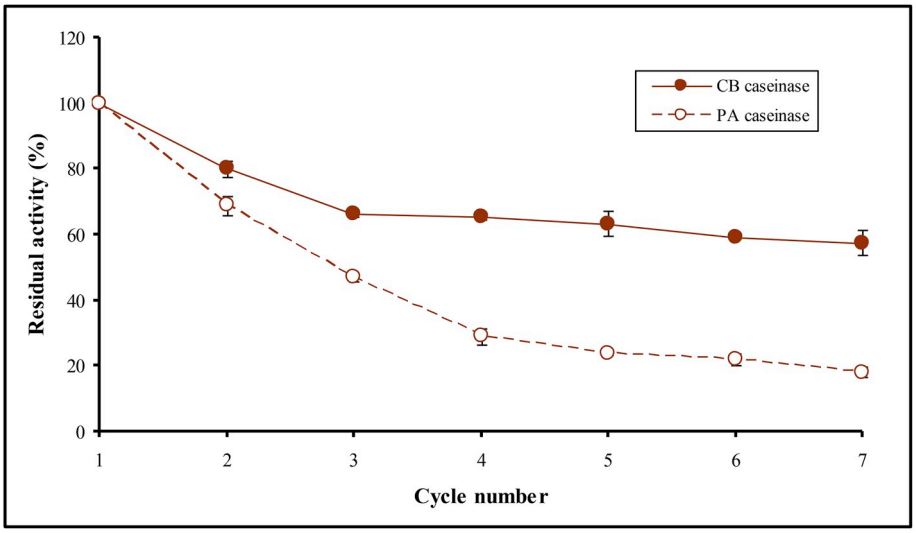

Fig. 11. Reusability of CB and PA caseinase.

et al. (2017) reported that negative ΔS° indicates that there is more stability in the deactivation of the complex linked by covalent binding.

3.4.6. Stability in presence of some additives and inhibitors

The results presented in Fig. 10 revealed that PA caseinase was more compatible than CB caseinase to SDS, tween 40, triton x-100 and urea. Significant inhibition in activity of CB and PA caseinase was observed in presence of EDTA and H_2O_2. Tween 40 and triton x-100 activated PA caseinase by 88 and 47.3%, respectivelt. However, they inhibited the CB caseinase by 33.6 and 68.2%. PA caseinase was considered as a prominent candidate for detergent formulation as bio-additive.

3.4.7. Operational stability (Reusability)

Enzyme reusability is important parameter that should be considered for industrial application. They are fundamental keys for managing reuse of the enzyme over a long period. To our knowledge this is the first time GC particles were used to get stable CB and PA caseinase. A primary objective in immobilized enzyme technology is to increase enzyme stability, since stability directly affects costs (Ricardia et al., 2018). Upon repeated use, gradual decrease in activity was observed (Fig. 11). After 5 cycles the CB caseinase retained 63.2% of its initial activity which was higher than that retained from PA caseinase (23.7%). The loss of activity may be due loss of a part of particles suspension being washed out each time (Sinha and Khare, 2015). CB caseinase was highly operational stable up to 7 cycles with residual activity (57.5%). Multipoint covalent binding of enzymes on activated supports promotes a rigidification of its structure and reusability. PA caseinase yielded lower residual activity (%) than CB caseinase possibly due to excess leaching of the enzyme due to weaker binding forces. It should be noted that the high operational stability could reduce the operation cost in industrial applications (Shojaei et al., 2017).

4. Conclusion

Modern science and technology always require new materials with special properties to achieve breathtaking innovations. Glass (G) and glass–ceramics (GC) which produced from raw materials (basalt) are cheaper than those from the elemental technical grade oxide powders and are used in industrial applications as they are abrasion and corrosion resistant. Caseinase enzyme was immobilized by covalent binding (CB) and physical adsorption (PA) on novel support. To our knowledge this is the first time GC particles were used to get stable CB and PA caseinase. A physicochemical characterization of the GC (particle size ≥ 1.0 mm) support was performed by XRD and SEM. One-factor-at-a-time experiments and the response surface methodology were used to optimize the immobilization conditions for the maximum

immobilization yield. Under optimized conditions the immobilization yield (IY %) was increased by 2.4 and 1.6-fold, respectively for PA and CB caseinase. The CB caseinase showed an improvement in thermal stability (higher *Ea*, $t_{1/2}$, *D*-value, *Ed*) with a great potential for reusability (7 cycles with 57.5% residual activity). Decreased *Km* of the PA caseinase indicates the increased its affinity to the substrate. Comparatively, covalent immobilization can eliminate or significantly reduce leaching of enzyme and can increase stability. The thermodynamic parameters for irreversible inactivation indicated that enthalpy (ΔH°), Gibbs free energy (ΔG°) and entropy (ΔS°) were higher for CB caseinase than that for PA caseinase within 50–70 °C. Regarding the immobilization process, our results demonstrated that the immobilized caseinase developed in this study was effective and provide a useful technique to increase enzymatic stability and practical applications in an industrial scale setting.

Conflicts of interest

There is no conflict of interests regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101028.

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