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Research article

Preparation and characterization of sugilite glass from basalt for α-amylase immobilization, statistical optimization of the immobilization process and description of free and immobilized enzyme

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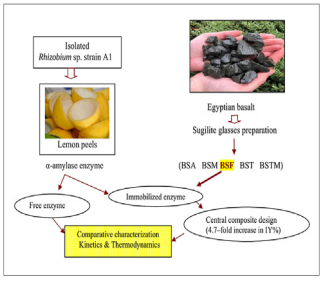
HIGHLIGHTS

- Lemon peels induced α-amylase production by isolated *Rhizobium* sp. strain A1.
- Using basalt as raw material for sugilite glass synthesis as new immobilization carriers.
- Sugilite BSF glass the suitable carrier was characterized by DSC, FTIR and SEM.
- Central composite design increased immobilization yield by 4.7–fold.
- Thermal and thermodynamic properties emphasize increased stability upon immobilization.

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial α-amylase was immobilized on sugilite from modified basalt rock as a new carrier. A set of glass compositions based on sugilite formula $KNa_2M_2Li_3Si_{12}O_{30}$ (M = Al or Mn or Fe) were prepared. The glasses were prepared through melting–quenching technique and samples of glass were converted to glass ceramic. Among the tested glasses and glass ceramic only sugilite glass based on M = Fe (BSF) give promising results. The sugilite BSF glass was characterized using DSC analysis, FTIR absorption, and SEM. The sugilite glass revealed high thermal resistant till ~770 °C. Under optimized conditions of the Central composite design, the immobilization yield improved by 4.7-fold. The affinity to starch increased after enzyme immobilization by 4.3-fold. The lower rate of deactivation constant and the increase of $t_{½}$ and *D*-value confirm the suitability of BSF and immobilization method in enhancing enzyme stability. The improvement in thermostability of immobilized α-amylase was judged by the change in thermodynamic parameters. In conclusion, the prepared sugilite BSF glass can be utilized as a new carrier suitable for stabilization of α-amylase enzyme by immobilization.

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1. Introduction

Scientists have been interested in using enzymes in various industries instead of chemicals. Microbial enzymes are preferred because they have the most desired properties for their applications in biotechnology (Abdel Wahab and Ahmed, 2018). Alpha-amylase is one of the most valuable enzymes as it is used in many applications such as in food, medicinal, pharmaceutical, clinical, and analytical chemistry. The production cost of microbial enzymes can be lowered by the use of cheap industrial agricultural-wastes (IAW) as sole carbon sources in the growth medium (Saha and Mazumdar, 2019). Technical and economic attention is paid to the IAW due to its low price in addition to its tendency to control and pollute the environment (Pal and Khanum, 2011). Lemon (*Citrus limon*) is the third most important species of citrus after orange and mandarin (Janati et al., 2012). Lemon peel is a by-product of lemon juice processing, with a high potential use.

For industrial uses of enzymes the main criteria are cost, stability, and their thermodynamic properties under harsh conditions (Pal and Khanum, 2011).

Immobilization of enzyme is an effective mechanism that stabilizes enzymes under process conditions. Enzyme immobilization can be defined as enzyme binding to carrier resulting lowering or loss its movement while retaining its catalytic activity. Immobilization systems allow us to offer stability, privacy, better activity and higher resistance to denaturation, facilitating diversified use in biotechnology fields (Pandey et al., 2017; Rodrigues et al., 2017; Chauhan and Upadhyay, 2021). The efficiency of immobilization depends on the immobilization method used and the choice of carrier (Chauhan and Upadhyay, 2021). Enzyme immobilization can involve different carriers (organic and inorganic) due to their chemical constitutes and different methods as cross-linking, physical adsorption, entrapment, and covalent binding (Misson et al., 2015).

Immobilization by physical adsorption technique is cheap, simple, as well as adsorbed enzyme retains high catalytic activity so, it is more commonly used than other methods (Eş et al., 2015). This method involves binding the enzyme to the carrier surface mainly through electrostatic force as hydrogen bonding, hydrophilic/hydrophobic, Van der Waals, and ionic bonding (Misson et al., 2015; Zdarta et al., 2018). Although these forces are weak, they are large enough in number to be capable of a reasonable binding.

In general, a carrier is considered ideal when it is low cost, high physical and chemical stability, good biocompatibility, and having many points for enzyme attachment (Remonatto et al., 2021). Inorganic carriers are proper for industrial applications because they are inert, affordable, non-toxic, insoluble, have physical strength, regeneration, stability, ability to increase enzyme activity/specificity and diminish product inhibition (Datta et al., 2013; Ahmed et al., 2018; Ricardia et al., 2018). Glasses have new applications due to their amorphous structure that causes great chemical stability as well as the ability to control chemical degradation and particle size (Ahmed et al., 2018). Glass is an amorphous solid prepared by rapid melt quenching technique. Immobilization on glass beads is an easy method because they are very stable, non-toxic, provide better handling, and can be retrieved easily from the mixture and washed for reuse (Chauhan and Upadhyay, 2021). The preparation of glass from raw materials is of great scientific, economic and technological importance. Glass produced using basalt as raw material, metallurgical slag, and fly ash is lower price than glass produced from elementary technical grade oxide powders (Khater et al., 2012). Basalt is a volcanic rock, fine-grained, and dark-colored that with lava flow appears as a rock. It consisted from about ten different oxides, thus designing a specific glass composition from basalt is difficult and accurate process (Ediz et al., 2021; El-Shennawi et al., 2007). Due to the characteristics of basalt glass, abrasion and a corrosion resistant tile made of basalt glass are used widely in industry.

Table 1. Chemical composition of the prepared sugilite glasses in wt%.

Oxide	SiO_2	Al_2O_3	TiO_2	Fe_2O_3	CaO	MgO	Na_2O	K_2O	MnO	Li_2O
BSA	50.06	14.31	1.93	14.50	8.91	4.28	2.71	1.60	0.16	1.53
BSM	48.86	11.79	1.88	14.15	8.69	4.18	2.64	1.56	4.71	1.49
BSF	51.19	12.36	1.97	14.83	9.11	4.37	2.76	1.64	0.18	1.56
BSTM	50.45	12.17	1.94	14.61	8.98	4.31	2.73	1.61	1.62	1.53
BST	47.09	14.97	2.39	17.97	11.04	5.31	3.35	1.98	1.99	1.89

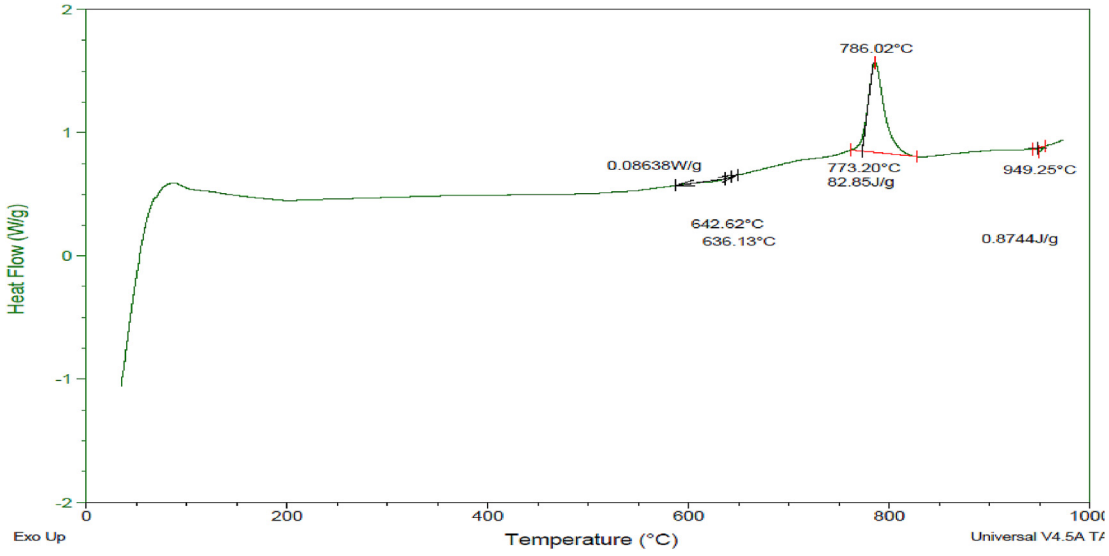


Figure 1. Differential Scanning Calorimetric analysis (DSC) of prepared sugilite BSF glass.

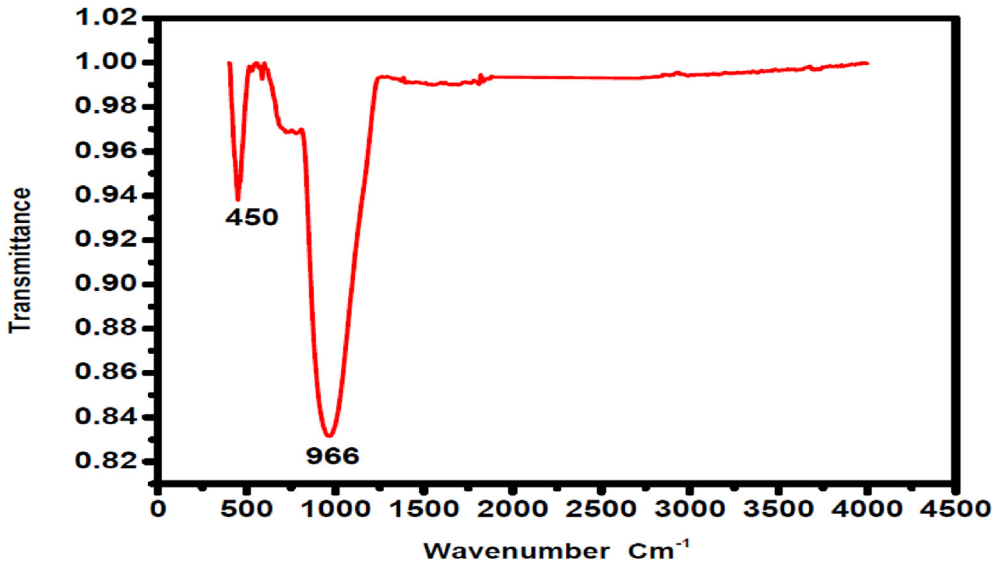


Figure 2. Fourier Transform Infrared absorption (FTIR) for prepared sugilite BSF glass.

Some factors affect the immobilization yield (IY%), to optimize the immobilization process, most of the studies changed one factor separately at a time. The design of the Central composite (Cc) is an efficient method that saves time and effort and provides the interaction of factors (Eslamipour and Hejazi, 2016).

A change in the kinetic and thermodynamic parameters of enzymes upon immobilization leads to a change in their catalytic reaction (Ricardia et al., 2018). Thermodynamic stability includes resistance to denaturation of the folded enzyme protein. Thermodynamic parameters (enthalpy, entropy, and free energy) are ideal indicators for the potential applications of enzyme in biotechnology (Zaboli et al., 2019).

In this paper modification of Egyptian basalt composition via adding definite quantity from SiO_2, Al_2O_3, K_2O and Li_2O was done to get new glass composition based on sugilite composition. The prepared sugilite glasses were characterized using DSC analysis, FTIR, and SEM. The prepared glasses were tested as novel immobilization carriers for α-amylase enzyme from the isolated bacterial strain (*Rhizobium sp*. strain A1, accession number OL655453). Central composite design was used to immobilization process optimization for the highest IY%. Additionally, a comparative study between free and immobilized enzyme on sugilite BSF glasses was performed.

2. Materials and methods

2.1. Materials

All chemical reagents used were of analytical grade.

2.2. Methods

2.2.1. Collection and preparation of lemon peel

Lemon peel was collected and washed (to remove dust) with H_2O, cut or crushed to small pieces (~1 cm) and was dried at 50 °C for 24 h in an oven. The dried sample was separated by 1 cm sieve and was packed in air-tight containers for use in the production medium.

2.2.2. α-amylase enzyme production by Rhizobium sp. strain A1 under submerged fermentation

It was performed in Erlenmeyer flasks (250 mL) containing 1 g of lemon peel and 50 mL of the production medium containing (g/L): bakers yeast 10, $(NH_4)_2SO_4$ 10, glucose 10, $CuSO_4$ 0.2, and Tween-80 (1 mL/L). Flasks were autoclaved at 121 °C for 20 min, after cooling, each flask was inoculated with 1 mL of cell suspension (1 day-old in 10 mL

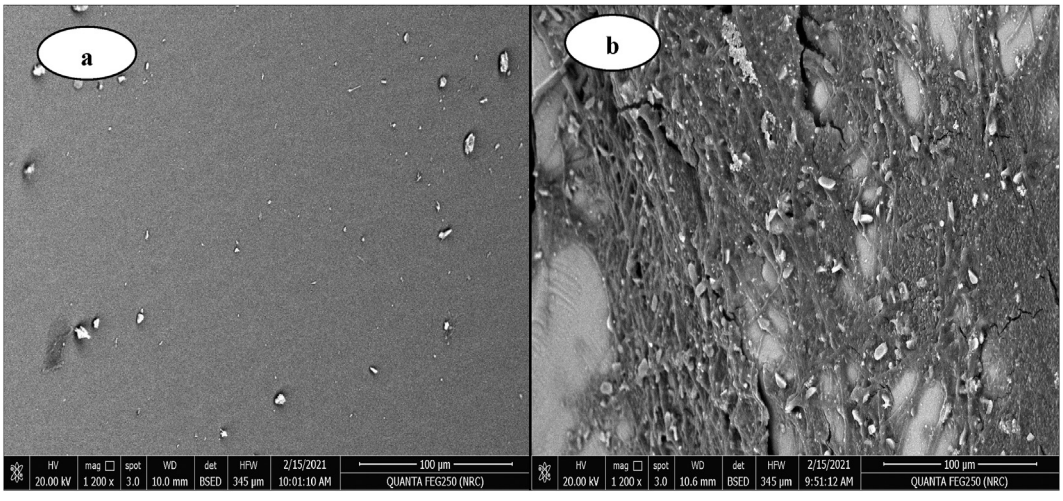


Figure 3. Scanning Electron Microscope (SEM) images of prepared sugilite BSF glass (a) before immobilization and (b) after immobilization with α-amylase enzyme.

Table 2. Central composite (Cc) design for *Rhizobium* sp. strain A1 α-amylase enzyme immobilization.

Run	Factor 1 A: amylase units [U]	Factor 2 B: carrier weight [mg]	Factor 3 C: loading time [h]	Immobilization yield [%]
1	1.750	100.000	12.000	30.66
2	1.125	175.000	36.136	70
3	1.125	175.000	5.864	26.43
4	1.125	175.000	21.000	70.55
5	0.500	100.000	30.000	35.88
6	1.125	175.000	21.000	70.55
7	1.750	100.000	30.000	65.45
8	0.500	100.000	12.000	14.25
9	1.125	301.134	21.000	40
10	0.500	250.000	30.000	10.12
11	1.750	250.000	30.000	52.79
12	1.125	175.000	21.000	70.55
13	1.125	175.000	21.000	70.55
14	1.750	250.000	12.000	40.25
15	0.074	175.000	21.000	0
16	1.125	175.000	21.000	70.55
17	0.500	250.000	12.000	30.77
18	1.125	175.000	21.000	70.55
19	1.125	48.866	21.000	35.88
20	2.176	175.000	21.000	50.66

sterile distilled water) of the isolated bacterial strain (*Rhizobium sp*. strain A1, accession number OL655453). Then the flasks were incubated for 7 days at 35 °C and 150 rpm. Finally, biomass was separated by centrifugation at (8000 xg, and 4 °C for 15 min) to obtain the clear culture filtrate.

2.2.3. Sugilite glass preparation

As a beginning material, Egyptian basalt (from Abu-Zaabal, Northeast of Cairo E, 30° 14′ 29.4″ N, 31° 24′ 39.6″) was employed. SiO_2, K_2CO_3, Al_2O_3, and Li_2CO_3 were used in small quantities. In an electrically heated Globar furnace, 100 g of the batch components were well mixed before being melted in a platinum crucible. At 1500 °C, the batches were heated at a rate of 600 °C/h until they melted. To ensure complete homogeneity, the melt was kept for 0.5 h at lower temperature with intermittent agitation. The glass melts were cooled by pouring on stainless plate at room temperature.

2.2.4. Converting sugilite glass into sugilite glass ceramic

To transfer the prepared glasses into glass ceramic (crystalline form), heat treatment of the prepared glasses at different temperature program was applied. According to DSC results we choose three heat treatment programs, one at 750 °C/2 h and 850 °C/2 h then at 950 °C/2 h with heating rate 10 °C/min to study the effect of heat treatment temperature on the crystallization process, phases developed and microstructure.

2.2.5. α-amylase enzyme immobilization with sugilite glass (and glass ceramic)

The produced α-amylase enzyme was immobilized by physical adsorption using the prepared sugilite glass and glass ceramic (as a new carrier) according to (Gashtasbi et al., 2014) method. Sugilite glass or glass ceramic (100 mg) with 1.0 mm particle size was suspended in sodium acetate buffer (0.25 ml, 0.05 M pH 5.0) containing 0.75 U of α-amylase enzyme at 4 °C for 24 h. The unbound enzyme was removed by washing with the same buffer. The IY% and the residual activity (RA%) were calculated using the following formulas (1) and (2) as suggested by (Rodrigues et al., 2017):

$$IY\% = (\text{Immobilized enzyme activity/Activity offered for immobilization}) \times 100 \quad (1)$$

$$RA\% = (\text{Obtained activity/ Initial activity}) \times 100 \quad (2)$$

2.2.6. Estimate α-amylase enzyme activity

The activity was performed by adding 0.5 mL enzyme solution or a specific weight of the immobilized enzyme to 0.5 mL starch solution (1%) dissolved in sodium acetate buffer 0.05 M, pH 5.0 (Sajjad and Choudhry 2012). The mixture was incubated for 20 min at 40 °C and the reducing sugar were determined according to (Mutrej et al., 2011) method. One enzyme activity unit is known as the amount of enzyme that released one μ mole of reducing sugar as glucose/min under the assay conditions. All experiments were performed in triplicate and results were expressed as mean values ± SD.

2.2.7. Characterization of synthesized sugilite glass

Differential Scanning Calorimetric analysis (DSC), using SDTQ600 instrument under inert gas was utilized to determine the temperatures of the glass transition (Tg) and crystallization (Tc) of the glass samples. The heating rate was 10 °C/min and alumina was used as blank reference material.

The Fourier transform infrared absorption (FTIR) for the prepared sugilite glasses were measured at room temperature in the range 4000–400/cm by an infrared spectrophotometer type (JASCO FT/IR-300E spectrophotometer, Japan), using the KBr disc technique. Two milligrams of powdered glass was mixed with 200 mg of KBr and the mixture was subjected to a load of 5 tons/cm^2 in order to produce clear homogeneous discs. The infrared absorption spectra were measured immediately after preparing the desired discs.

The morphology of selected glass sample was examined with a Scanning Electron Microscope, SEM model Philips XL30, using an accelerating voltage of 30 K.V., magnification × 10 up to × 400,000, and resolution for wavelength (3.5 nm).

2.2.8. Optimization of α-amylase enzyme immobilization by central composite design (Cc)

In this step we optimized the immobilization process to improve the IY % by optimizing three factors, A: α-amylase enzyme loading units (U), B: carrier weight (mg) and C: loading time (h) by Cc design giving 20 run as shown in the table of design. The significance of the design was analyzed by ANOVA.

2.2.9. Characterization of α-amylase enzyme free and immobilized on sugilite glass

The optimum temperature for highest α-amylase enzyme (free and immobilized) activity was determined by measuring the activity at different temperatures (from 25 to 70 °C) in sodium acetate buffer (0.05 M, pH 5.0). The activation energy (E_a) was calculated as the following Eq. (3) from the slope of the Arrhenius plot:

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

where, R is the gas constant (8.314 kJ/mol), and T is the absolute temperature (°K).

For optimum substrate concentration, α-amylase enzyme (free and immobilized) activity was determined by carrying out the reaction at different starch concentrations from 0.25 to 3 (g%) in 0.05 M sodium acetate buffer pH 5.0. The Michael's constant K_m and the maximum velocity V_{max} are important kinetic constants that determine enzyme sensitivity and were estimated using the plot of Lineweaver and Burk (1934).

The impact of metal ions on the activity of α-amylase enzyme (free and immobilized) was studied by pre-incubating with 5 mM of the tested metal ions (Al^{2+}, Ba^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, K^+, Mg^{2+}, Mn^{2+}, Na^+, Ni^+, and Zn^{2+}) at 25 °C for 0.5 h before adding the substrate. Then the

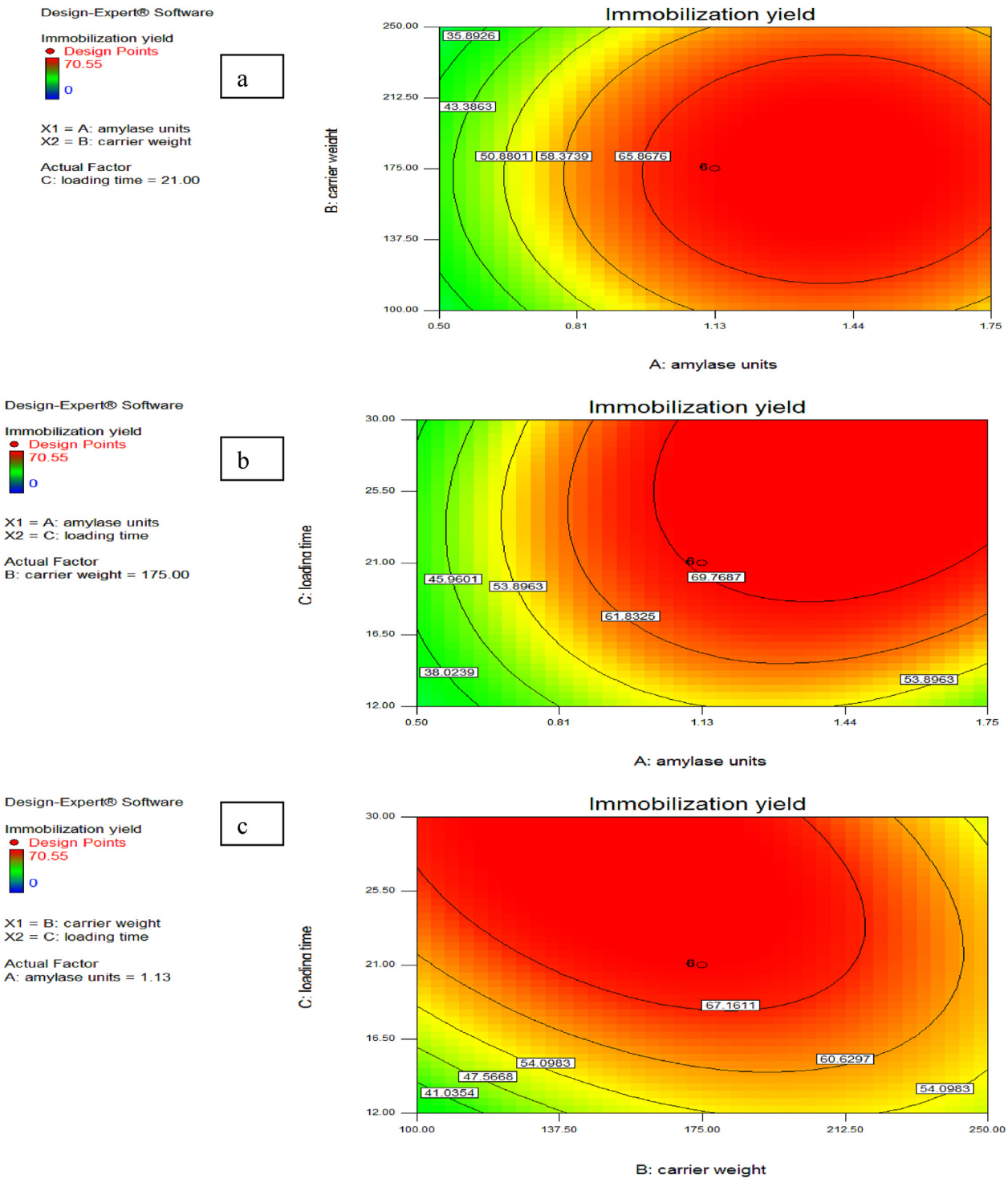


Figure 4. Contour plot showing an interaction between (a) carrier weight and α-amylase enzyme loading units, (b) loading time and α-amylase enzyme loading units, and (c) loading time and carrier weight.

reaction was carried out under optimum conditions and the relative activity was determined as follows in Eq. (4) (α-amylase enzyme activity in the absence of metal ion was 100%):

$$\text{Relative activity (\%)} = (\text{Final activity/ initial activity}) \times 100 \quad (4)$$

2.2.10. *Thermal stability and thermodynamic characterization*

The thermostability was studied by incubating α-amylase enzyme (free and immobilized) for 60 min at specific temperatures (40, 50 and 60 °C). Samples were taken at 15 min intervals and determine the residual activity. The α-amylase enzyme thermal and thermodynamic parameters were determined as presented in Eqs. (5), (6), (7), (8), (9), (10), and (11). Denaturation rate constant (k_d/min) was determined from the Arrhenius plot of log residual activity (%) versus time (min) as follows in Eq. (5):

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

The activation energy (E_d KJ/mol) for α-amylase enzyme denaturation was determined by an Arrhenius plot of log denaturation rate

Table 3. Statistical analysis (ANOVA) for α-amylase immobilization by Cc design.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	9912.018	9	1101.335	42.3872	<0.0001	significant
A-amylase units	2461.017	1	2461.017	94.7174	<0.0001	
B-carrier weight	2.120203	1	2.120203	0.0816	0.7810	
C-loading time	1082.466	1	1082.466	41.66099	<0.0001	
AB	4.758612	1	4.758612	0.183145	0.6778	
AC	268.5403	1	268.5403	10.33534	0.0093	
BC	520.5151	1	520.5151	20.03312	0.0012	
A2	3694.812	1	3694.812	142.2026	<0.0001	
B2	1923.721	1	1923.721	74.03843	<0.0001	
C2	904.1735	1	904.1735	34.79901	0.0002	
Residual	259.8273	10	25.98273			
Lack of Fit	259.8273	5	51.96547			
Pure Error	0	5	0			
Cor Total	10171.85	19				

R^2 0.974, adj R^2 0.951, pred R^2 0.798.

constant (ln *k*d) versus reciprocal of the absolute temperature (°K) using the following Eq. (6):

$$\text{Slope} = -E_d/R \quad (6)$$

Half-life ($t_{½}$ min) and D-values (min) for thermal inactivation were given by the following Eqs. (7) and (8):

$$t_{½} = \ln 2/k_d \quad (7)$$

$$D = \ln 10/k_d \quad (8)$$

The change in thermal parameters, enthalpy (ΔH*, kJ/mol), entropy (ΔS*, J/mol/K), and free energy (ΔG* kJ/mol) of α-amylase enzyme was determined using the following Eqs. (9, 10, and 11):

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

$$\Delta G^* = -RT \ln (k_d.h/k_b.T) \quad (10)$$

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

where: h (Planck constant, 11.04×10^{-36} J min) and K_b (Boltzman constant, 1.38×10^{-23} J/K).

3. Results and discussions

3.1. Sugilite glass preparation

A set of glass compositions base on sugilite formula, $KNa_2M_2Li_3Si_{12}O_{30}$ (M = Al or Mn or Fe), were designed. The glasses were denoted as BSA, BSF, BSM, BST and BSTM; where A = Al, F=Fe, M = Mn, and T = triplet addition of Al, Fe and Mn. The Chemical compositions of the prepared glasses in wt% are illustrated in Table 1. Samples of glasses were converted to sugilite glass ceramic. Sugilite glasses and glass ceramic were tested as immobilization carrier for α-amylase enzyme.

3.2. α-amylase enzyme production

Lemon peel induced α-amylase enzyme production by isolated bacterial strain *Rhizobium* sp. strain A1 under submerged fermentation with 1.084 U/mL. The recycling of IAW which is widely available as nutritional sources for enzymes production plays an essential role in reducing the production charge and solving the pollution problem (Ahmed et al., 2019). Lemon peel is a by-product of lemon juice processing with a high potential use (Janati et al., 2012). The chemical properties of lemon peel (%) are: humidity 60.96, carbohydrate 30.74, protein 3.59, lipid 2.93, ash 1.78, fiber 4.78%, and vitamin C (5.79 mg/g) as reported by (Pham et al., 2020). α-amylase enzyme was produced by bacterial isolate *Bacillus cereus* amy3 using mosambi peel as cheep agro-residues (Saha and Mazumdar, 2019).

3.3. α-amylase enzyme immobilization with sugilite glass

The produced α-amylase enzyme was immobilized by physical adsorption on all the prepared sugilite glass and glass ceramic as new carrier. The results showed that only sugilite BSF glass was able to physically absorb α-amylase enzyme with IY (15%). The failure of enzyme immobilization on the other prepared sugilite glass (BSA, MSM, MST and BSTM) may be due to the inhibitory effect of the minerals present as Al^{2+} and Mn^{2+}. Thus, the focus in this paper has been on the characterization of sugilite BSF glass and the statistical enhancement of IY%.

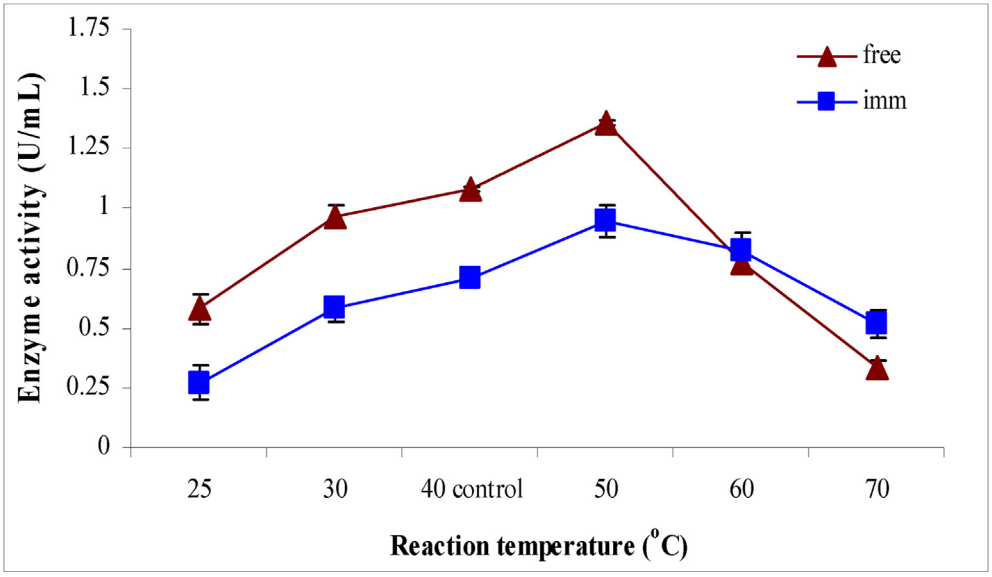


Figure 5. Effect of temperature on the activity of free and immobilized α-amylase on sugilite BSF glass.

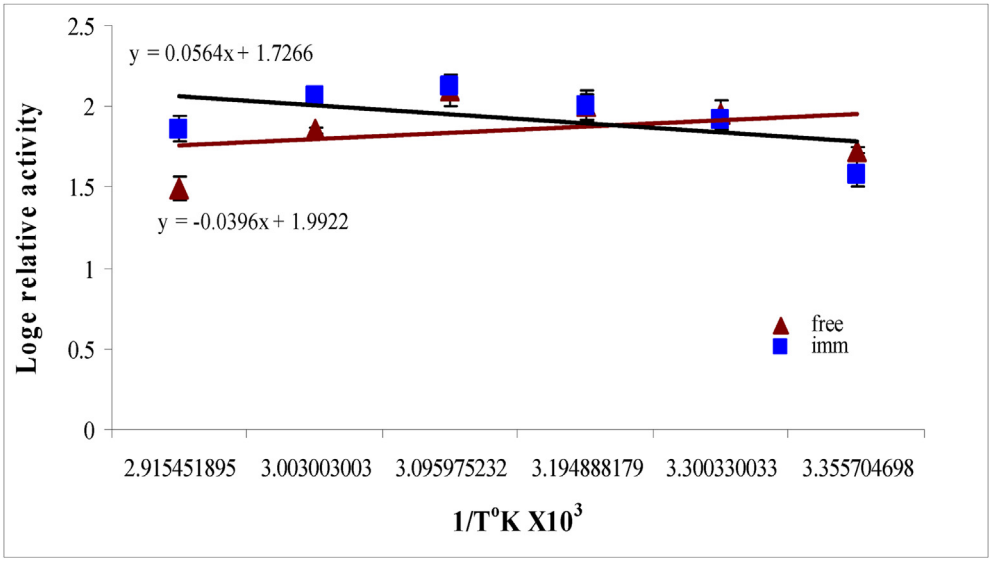


Figure 6. Arrhenius plot for temperature dependence of the activity of free and immobilized α-amylase enzyme on sugilite BSF glass.

3.4. Characterization of synthesized sugilite BSF glass

DSC thermogram for the prepared sugilite BSF glass sample was illustrated in Figure 1. DSC analysis depicted endothermic effect revealed to glass transition temperature (Tg) at 643 °C followed by significant exothermic peak related to crystallization temperature (Tc) at 786 °C. At Tg the viscosity was slightly decreased to about 10^{12} Pa allowing to more mobility of glass ions to get more ordering structures and development of tiny crystals (nuclei). On the other hands maximum crystal growth was reached at crystallization temperature (Tc) (Abdel-Hameed and Elwan, 2012). From these results we can conclude that, the prepared sugilite BSF glass have thermal stability till ~770 °C, i.e., it can resist temperature to about 635 °C. This property make this glass can be used under hard conditions in different applications.

Analysis using Fourier transform infrared spectroscopy (FTIR) is a technique for determining the molecular components and structures of materials based on their electromagnetic spectrum wave of absorption or emission, whether they be solids, liquids, or gases (Prasath et al., 2020). Infrared spectroscopy can also be used to determine the chemical bonding of substances for qualitative and quantitative examination. The FTIR spectra of sugilite BSF glass powder are shown in Figure 2. The X-axis shows wave number, whereas the Y-axis represents light transmittance, the wave number interval is 4000–500/cm. The signal at 450/cm are attributed to the bending vibration of (Si–O–Si) while the broad band centered at 966/cm is attributed to asymmetric stretching frequency of Si–O–Si (Saraya, 2011), this results revealed that the building unit in this glass is SiO_4 due to the presence of SiO_2 with high quantity.

Figure 3 revealed a comparison between prepared sugilite BSF glass (before and after) immobilization with enzyme using SEM. Prepared sugilite BSF glass (Figure 3a) shown smooth surface does not have any distinct composition which is the normal feature of amorphous structure. On the other hand, after fixation (Figure 3b), a thick layer of crosslinking enzyme appears covering the smooth surface of sugilite BSF glass, which indicates the success of the immobilization process.

3.5. Optimization of α-amylase enzyme immobilization by central composite (Cc) design

As shown in Table 2 and Figure 4 (a, b, c) the interaction between α-amylase enzyme loading (U), carrier weight (mg) and loading time (h) led to remarkable variation in immobilization yield from 0 (run 15) to 70.55% in central runs (4, 6, 12, 13, 16, 18) causing 4.7 –fold increase compared to un-optimized conditions (IY 15% using 100 mg carrier, 0.75 U of enzyme at loading time 24 h).

The Immobilization yield (IY%) can be calculated from the following Eq. (12):

$$\text{IY}\ (\%) = +70.55 + 13.42*\text{α-amylase enzyme loading units} - 0.39*\text{carrier weight} + 8.90*\text{loading time} + 0.77*\text{α-amylase enzyme loading units}*\text{carrier weight} + 5.79*\text{α-amylase enzyme loading units}*\text{loading time} - 8.07*\text{carrier weight}*\text{loading time} - 16.01*\text{α-amylase enzyme loading units}^2 - 11.55 * \text{carrier weight}^2 - 7.92 * \text{loading time}^2 \quad (12)$$

Lower IY (57.1%) obtained at optimum immobilization conditions for α-amylase enzyme and chitosan-Fe_3O_4 support (Bindu et al., 2018).

The success of the Cc design was emphasized by statistical analysis (ANOVA) as shown in Table 3. The model F-value of 42.39 indicated that the model is significant. As the value of R^2 is close to 1 as the success of the design is indicates as in our case (0.974). Moreover, the values of adj R^2 0.951 and pred R^2 0.798 are in reasonable agreement which implicate the design success.

3.6. Characterization of α-amylase enzyme free and immobilized on sugilite BSF glass (A-BSF)

The optimum temperature for α-amylase enzyme catalytic activity of both free and A-BSF enzyme was seen at 50 °C (Figure 5). Similarly (Zusfahair et al., 2020), noted that there is no change in the optimum reaction temperature (40 °C) before and after immobilization of bacterial amylase. By raising the temperature to 70 °C, there is an obvious loss of free enzyme activity by 3.2-fold which is higher than the loss of A-BSF activity (1.4-fold). These results confirm that immobilization of the enzyme on the sugilite BSF glass enhanced its stability at elevated temperatures. The immobilized α-amylase enzyme has higher stability than the free enzyme as reported by (Yandri et al., 2020).

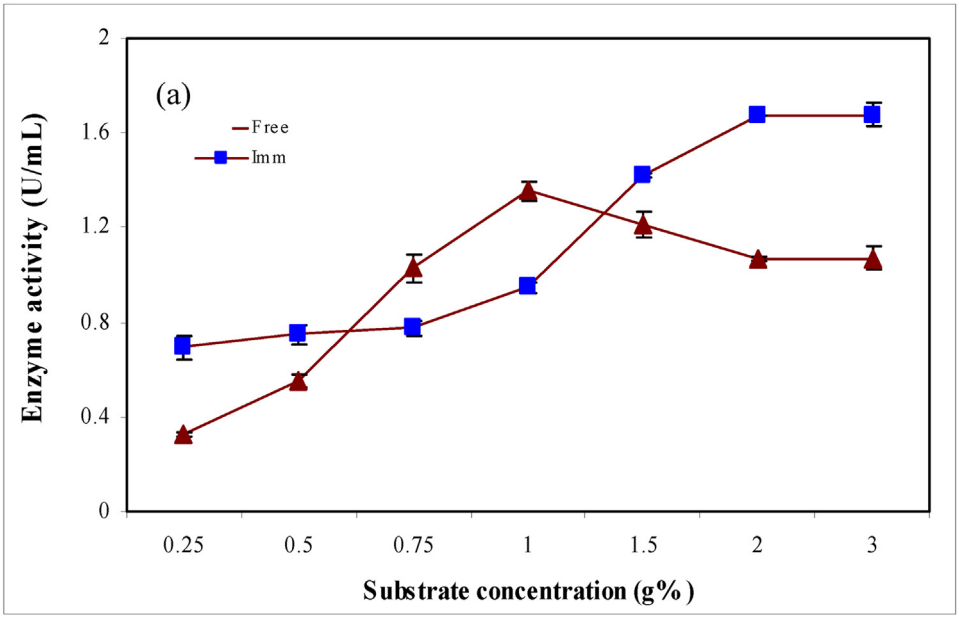
Activation energy of enzymatic reaction catalysis (E_a) is defined as the energy wanted to form a complex of enzyme-substrate, and a low E_a value reflects high enzyme catalytic efficiency. From the Arrhenius plot (Figure 6) E_a for the A-BSF enzyme (15.25 kJ/mol) was higher than that for the free one (10.25 kJ/mol) by 1.5-fold. These results indicated that immobilization reduced the enzyme catalytic efficiency by increasing the energy required to make the activated complex of enzyme-substrate. As reported by (Mohapatra, 2017) a 1.1-fold increase in E_a obtained after

enzyme immobilization. Similar result was observed by (Duman and Tekin, 2020) for E_a of the free enzyme (15 kJ/mol) which was lower than that of immobilized enzyme (102 kJ/mol) by 6.8 -fold.

Effect of substrate concentration on enzyme activity was illustrated in Figure 7 (a). As shown, the A-BSF enzyme had a higher substrate concentration (2%) for maximum activity (1.7 U/mL) compared to that of the free enzyme (1.4 U/mL) at 1% concentration. Investigation of enzyme kinetic parameters (K_m and V_{max}) is also an important feature to check the efficiency of the immobilization process, and was calculated from a Lineweaver–Burk plot (Figure 7b). K_m value gives an idea about the enzyme affinity to its substrate. The K_m of free and A-BSF were 15.4 and 3.6 (mg/mL), respectively, which clearly indicates an approximately 4.3-fold increase in its affinity after immobilization on sugilite BSF glass.

This may be due to the widening of the enzyme molecule on the surface of BSF particle with a good orientation that produces higher affinity for the substrate and more available active sites (Ahmed et al., 2019). Similar changes in K_m of amylase upon immobilization have been reported by (Zusfahair et al., 2020). On the contrary (Bindu et al., 2018; Yandri et al., 2020), indicated an increase in K_m for α-amylase enzyme after immobilization. The decrease of K_m value indicates a higher affinity for substrate and a lower substrate concentration is required to reach the maximum reaction rate which confirms the efficacy and efficiency of applied carrier and immobilization method. V_{max} is the maximum enzyme reaction rate.

The V_{max} value of the A-BSF enzyme was determined as 1.5 U/mL, which is 1.5-fold lower than the value of free α-amylase enzyme (2.3 U/mL). The decrease of V_{max} value can be attributed to some conformational changes in the enzyme molecule after immobilization as well as molecular crowding that occurs by the adsorption on the carrier (Yandri et al., 2020). Similarly (Antony et al., 2016), noted a decrease in V_{max} upon α-amylase enzyme immobilization by 2.5-fold, due to changes in enzyme conformations leading to a decrease in affinity to the substrate.



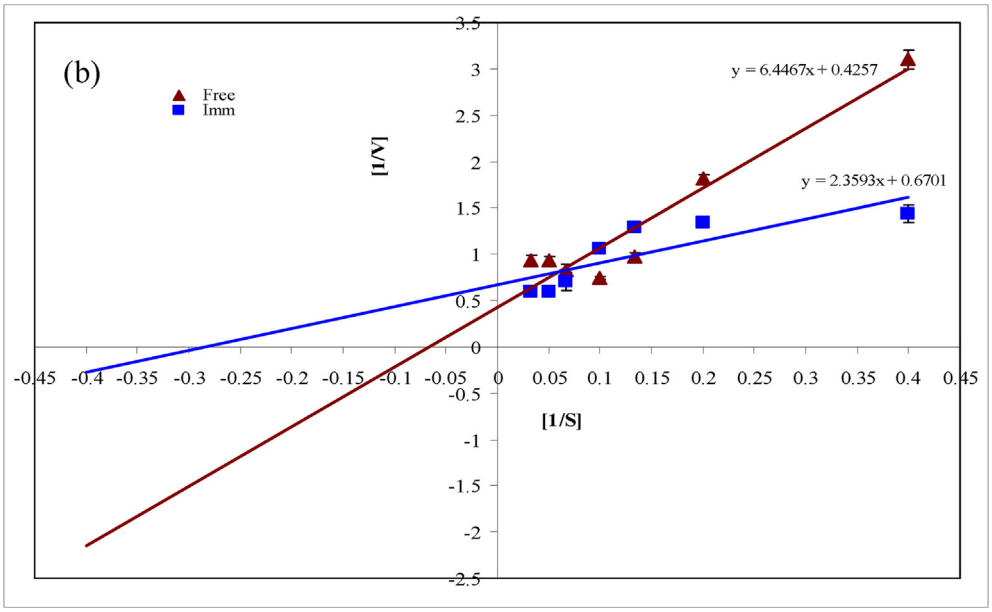


Figure 7. (a) Effect of substrate concentration on the α-amylase activity and (b) Lineweaver–Burk plot for the free and immobilized α-amylase enzyme on sugilite BSF glass.

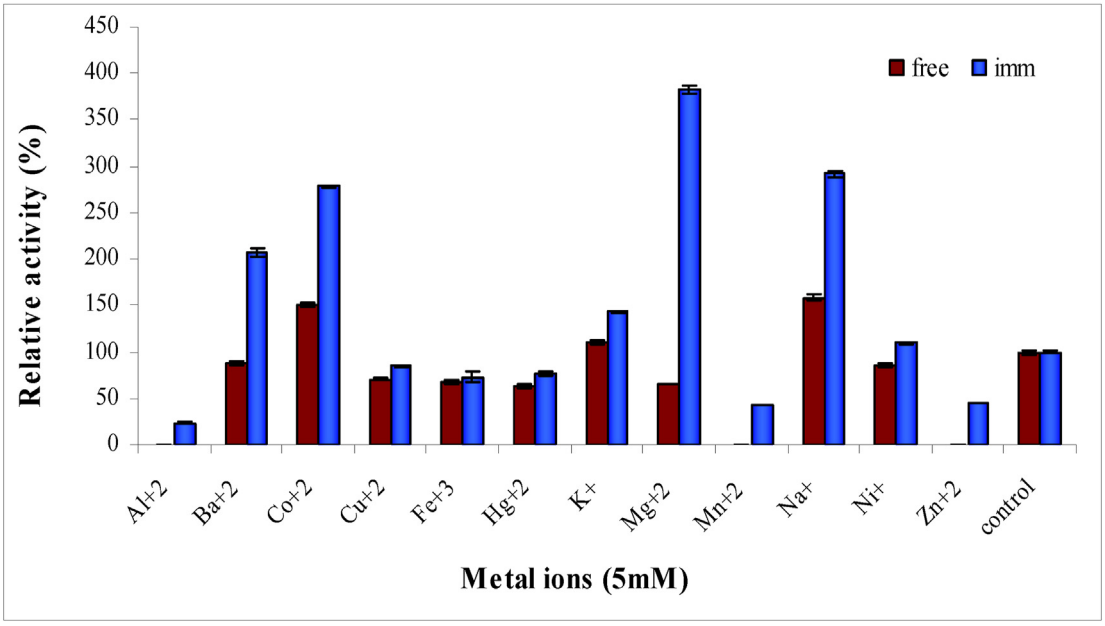


Figure 8. Effect of some metal ions on the activity of free and immobilized α-amylase enzyme on sugilite BSF glass.

The influence of some metal ions on the activity of free α-amylase enzyme and A-BSF was tested. As shown in Figure 8, the activity of A-BSF was enhanced in the presence of Mg^{+2} and Na^{+} to 383, and 291%, respectively. In addition, free enzyme is highly stable and activated in the presence of Na^{+} and Co^{+2}, retaining 159 and 150%, respectively. Similar results were reported by (Mohamed et al., 2014) about the effect of Co^{+2} on the free and immobilized α-amylase enzyme. In agree with our result (Khan et al., 2012) observed that both K^{+} and Na^{+} activated free α-amylase enzyme and A-BSF. In the presence of Zn^{+2}, A-BSF was inhibited by 54.3%, however free α-amylase enzyme was totally inactivated. On the other side, Cu^{+2} and Fe^{+2} ions reduced the activity of the free enzyme by 30 and 32%, and the A-BSF by 15 and 27%, respectively. Although, Al^{+2} and Mn^{+2} inhibited the free α-amylase enzyme completely, the A-BSF showed 24 and 43% relative activity, respectively. These results confirm that the failure to immobilize the α-amylase enzyme on the prepared sugilite glass (BSA, BSM, BST and BSTM) is due to the inhibitory effect of the minerals present (A: Al^{+2}, M: Mn^{+2}, T: Al^{+2}, Mn^{+2}, Fe^{+3}).

3.7. Evaluation of thermal stability and thermodynamic parameters

Thermal stability is an important and useful for the industrial application of microbial α-amylase enzyme. Thermal studies were conducted to evaluate the role of immobilization process in improving the stability of enzyme to thermal inactivation. As shown in Figure 9a, it is evident that the A-BSF enzyme has excellent resistance to heat inactivation. In general, this stability results from the molecular hardness produced by attachment to a solid carrier and generation protected microenvironment. After 0.5 h of incubation at 50 °C, the free and A-BSF enzyme retained 61.9 and 76.3% of the activity, respectively, indicating the positive role of sugilite BSF glass particles in protecting α-amylase activity at elevated temperatures.

The activation energy for thermal denaturation (E_d) is the minimum amount of energy needed to initiate the enzyme denaturation process. As calculated from Figure 9b, the E_d for A-BSF enzyme was found to be 68.26 kJ/mol which is higher than that of free enzyme (67.87 kJ/mol), indicating that A-BSF enzyme is more stable to heat inactivation. The higher value of E_d means that more energy is required to denature the enzyme. The increase of E_d for the enzyme after immobilization indicating that the enzyme structural conformation becomes more folding causing the enzyme structure is more rigid and less flexible, requiring higher energy to denature the enzyme (Yandri et al., 2020).

Thermal inactivation kinetic parameters (deactivation constant rate K_d, half life $t_{½}$, and D-value) of free α-amylase and A-BSF enzyme towards thermal processes were presented in Table 4. As seen from results, immobilization process protects the enzyme from thermal inactivation. At 60 °C, the k_d for the A-BSF enzyme was 9.1×10^{-3}/min, which is lower than that of the free enzyme (12.6×10^{-3}/min), confirming the suitability of the new carrier prepared from basalt as raw material. A lower k_d for the immobilized α-amylase enzyme indicates a lower rate of enzyme denaturation. On the contrary (Yandri et al., 2020), found that k_d of immobilized α-amylase enzyme was 3.8-fold higher than that of free form at 65 °C. The decrease k_d value is predicted because the enzyme is less flexible, so unfolding is decreased and enzyme stability is increased (Yandri et al., 2020). Based on Table 4, it is shown that the $t_{½}$ of the A-BSF enzyme has increased 1.3 and 1.4-fold at 40 and 60 °C, respectively compared to the free α-amylase enzyme. In addition, at 50 °C, D-value of A-BSF enzyme was calculated as 513.9 min, which is higher than its free form by 1.4-fold. Increasing the $t_{½}$ value and D-value of the α-amylase enzyme confirms the improvement of its thermal stability after immobilization Singh et al. (2019).

Behavioral performance of free α-amylase enzyme and A-BSF enzyme was examined and thermodynamic parameters (enthalpy, entropy and Gibbs free energy) which reflect the feasibility and spontaneous nature were determined. The enthalpy of activation for denaturation (ΔH^*) is a thermodynamic parameter that determines the total amount of energy needed for enzyme denaturation. Large and positive ΔH^* values are often associated with higher enzyme heat stability (Zaboli et al., 2019). The results showed that increasing the temperature decreased the values of ΔH^* of both free α-amylase enzyme and A-BSF enzyme gradually, indicating that at higher temperatures, lower energy was required for denaturation (Table 4).

Comparison of the ΔH^* values of free α-amylase enzyme and A-BSF enzyme indicates that immobilization leads to greater stability of the enzyme during thermal inactivation (increasing the energy required for inhibition). At 60 °C, the ΔH^* for A-BSF enzyme was 1.1-fold higher than that of the free α-amylase enzyme, which agrees with (Savitha et al., 2020). Increased ΔH^* values after enzyme immobilization as reported by Bedade et al. (2019) are associated with improved thermal stability as a result of modifications in the enzyme's secondary structure.

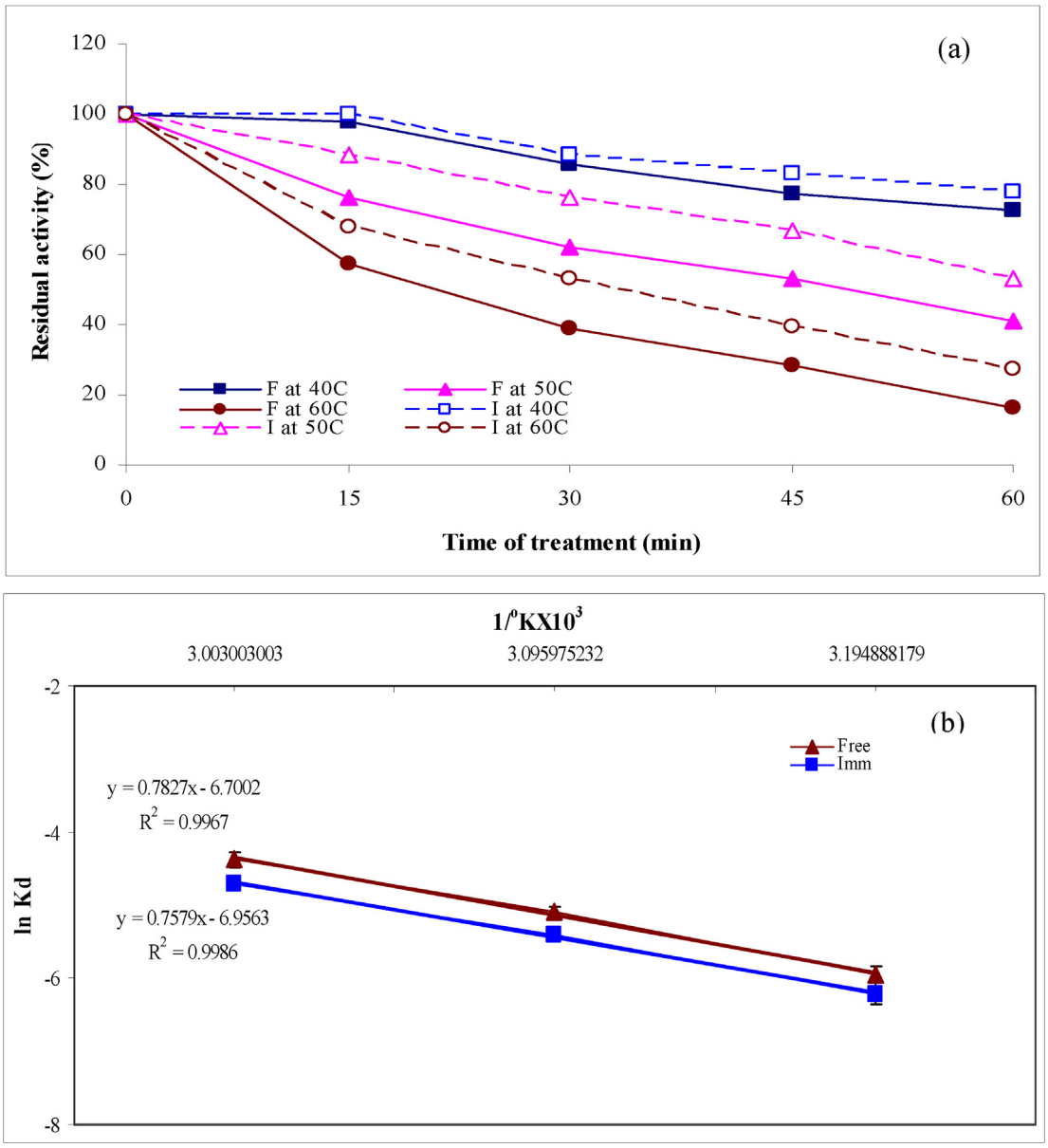


Figure 9. Thermal stability of free and immobilized α-amylase enzyme on sugilite BSF glass (a) and Arrhenius plot for the identification of activation energy for denaturation of free and immobilized α-amylase enzyme on sugilite BSF glass (b).

Gibbs free energy (ΔG*) is defined as an indicator of the energy required to cross the activation energy barrier for a reaction (Zaboli et al., 2019). As can be seen from the results, the ΔG* value for A-BSF enzyme is greater than that of the free α-amylase enzyme, which confirms that A-BSF enzyme needs more energy for thermal inactivation.

Savitha et al. (2020) observed a similar increase in ΔG* for α-amylase enzyme after immobilization. Bedade et al. (2019) reported that ΔG* values are directly related to the stability of the protein and thus to the higher thermal stability. Higher ΔG* for the immobilized enzymes shows that the thermal unfolding at high temperature is controlled by the immobilization process as well as increased thermal stability that provides resistance to heat denaturation (Zaboli et al., 2019).

Entropy (ΔS*) is the change upon protein folding in a disorder of the structure and has a direct relationship to enzyme stability. ΔS* is the extent of difference in local distortion of enzyme molecules between ground state and transition state (Bedade et al., 2019). The negative ΔS* values for the enzyme during thermal inactivation indicate a decrease in enzyme aggregation (Zaboli et al., 2019). The ΔS* values of A-BSF enzyme are lower compared to the free α-amylase enzyme indicating a decrease in the number of enzyme molecules in the case of transitional activation at the respective temperature. Similarly, Savitha et al. (2020) reported that immobilized α-amylase enzyme has lower ΔS* values compared to free α-amylase enzyme. Also, Karam et al. (2017) found that

Table 4. Kinetic and thermodynamic parameters for thermal inactivation of free and immobilized α-amylase enzyme.

Temperature (°C) Value	Free enzyme			Immobilized enzyme		
	40	50	60	40	50	60
Half life time ($t_{½}$ min)	264.3	111.7	55.2	346.6	154.7	76.1
Decimal reduction time (*D*-value min)	877.8	371.2	183.5	1151.3	513.9	252.9
Enthalpy (ΔH* kJ/mol)	65.27	65.19	65.10	65.66	65.57	65.49
Entropy (ΔG* J/mol K)	92.25	92.97	93.98	92.95	93.84	94.87
Free energy (ΔS* J/mol K)	−0.09	−0.10	−0.11	−0.10	−0.11	−0.11
Activation energy for denaturation (E_d kJ/mol)	67.87			68.25		

both free and immobilized amylase enzyme have negative ΔS* and the lower value of immobilized form indicates that it was more ordered compared to the free one.

4. Conclusion

Lemon peel induced α-amylase enzyme production by isolated bacterial strain *Rhizobium* sp. strain A1 under submerged fermentation. Sugilite glasses were prepared using modified basalt as raw material. α-amylase enzyme was successfully immobilized by physical adsorption on sugilite BSF glass (as a new carrier). Central composite design optimization increased the immobilization yield by 4.7–fold. Sugilite BSF glass was characterized by DSC thermogram, FTIR, and SEM. The immobilization of the α-amylase enzyme on the BSF improved its affinity for starch by 4.3-fold. Lower deactivation constant rate and higher ($t_{½}$ and D-value) confirms the suitability of the new carrier and the immobilization method in enhancing enzyme stability. In addition, the activation energy for enzyme denaturation for immobilized α-amylase enzyme was higher than that of free form, indicating its higher stability to heat inactivation. The change in thermodynamic parameters after immobilization confirms the enhancement of the thermal stability of α-amylase enzyme by immobilization. In future work, the authors will study the possibility of using the inactivated enzyme in some industrial processes.

Declarations

Author contribution statement

Salwa A.M. Abdel-Hameed: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Samia A. Ahmed: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Faten A. Mostafa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ola. N. Almasarawi: Performed the experiments.

Walaa A. Abdel Wahab: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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