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α-Amylase immobilization onto functionalized graphene nanosheets as scaffolds: Its characterization, kinetics and potential applications in starch based industries

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ABSTRACT

α-Amylase is imperative for starch and its deriviatized industries. Functionalized graphene sheets were tailored and optimized as scaffold for α-amylase immobilization using Response Surface Methodology based on Box–Behnken design, with an overall immobilization efficiency of 85.16%. Analysis of variance provided adequacy to the mathematical model for further studies. Native and immobilized functionalized graphene were characterized using transmission and scanning electron microscopy, followed by Fourier transform infrared (FTIR) spectroscopy. Wheat α-amylase conjugated with functionalized graphene sheets were visually evident on transmission and scanning micrographs while the FTIR spectra showed interplay of various chemical interactions and bonding, during and after immobilization. Optimum pH and optimum temperature for immobilized enzyme though remained unchanged but showed broader range whereas K_m showed a slight decrease (1.32 mg/mL). It also showed enhanced thermal and storage stability and retained 73% residual activity after 10 uses. These ensemble of properties and non-toxic nature of functionalized graphene, makes it viable to be absorbed commercially in starch processing industries.

1. Introduction

α-Amylase (EC 3.2.1.1), an endohydrolase, holding exquisite importance in a variety of starch based industries such as food, fermentation, textile, paper, detergent and sugar has quelled the chemical hydrolysis in starch processing industry [1–4]. It can be used in field related with biotechnology such as: removing environmental pollutant, conversion of starch to desired substrate by many microorganisms, infiltration of waste containing starch and production of biochemical material with the help of starch substrate (maltose, high fructose corn syrup, oligosaccharides mixtures, maltotetrose syrups, high molecular weight-branched dextrins, bio-alcohols) [5,6]. For the application in a flow-through system to analyze starch containing waste water, along with amyloglucosidase, α-amylase can be used [7].

Enzymes being nature's sustainable catalysts offer a mild, efficient and "green" process. However, the diversity of soluble α-amylase in industrial applications are modest with lack of long-term operational stability, difficult recovery and reusability of enzyme thus rendering their function cost intensive and uneconomical [8]. Many of these underlying constraints can be circumvented by immobilizing the soluble enzyme to some suitable support which offers commercial viability due to possible increase in stability, good catalytic activity, easier product and enzyme recovery, continuous operation of enzymatic processes, convenience in handling, reusability and reduced susceptibility to microbial contamination [9,10]. The properties of supported enzyme preparations, upon interfacing the biomolecules with different carriers, are governed by the properties of both the enzyme and the carrier material [11]. The interaction between the two provides immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties [9]. Recently nano-scaled materials have exhibited advantages over traditional bulk materials owning to their miniature size, large surface area and high enzyme loading

Abbreviations: RSM, response surface methodology; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; ANOVA, analysis of variance; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, Scanning Electron Microscopy; TEM, Transmission Electron Microscopy; FTIR, Fourier transform infrared spectroscopy

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capacity [12].

The outlook for green and sustainable catalysis with a rich polytypes of carbon like graphene presents a rich class of solid state materials that are non-polluting and reusable. Since, it can be derived chemically from graphite; it is an abundant and inexpensive natural source [13]. Graphene, one-atom-thick two-dimensional carbon nanomaterial with extraordinary electronic, thermal, and mechanical properties when combined with enormous surface area provides perfect platform for immobilizing biomolecules [14]. Being structurally composed of carbon atoms, it does not modify native biochemical properties of attached biomolecules significantly. With the rapid development of synthesis and functionalization approaches, graphene and its related derivatives have shown outstanding potentials in many fields, such as nanoelectronics, composite materials, sensors, drug delivery and catalysis [15]. Graphene oxide biocompatibility on animal models have exhibited non-toxic effects of the material under low dose administration [16,17].

Optimization is the crucial step to find out the best operating conditions and maximize the desired responses in any experimental subject [18]. Response surface methodology (RSM) is a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables [19]. These methods allow the development of mathematical models that permit assessment of the relevance as well as statistical significance of the factor effects being studied as well as evaluate the interaction effects between the factors [20]. The optimization process involves evaluating the response of the statistically designed combinations, estimating the coefficients by fitting the experimental data to the response function, predicting the response of the fitted model, and checking the adequacy of the model [21].

The present study aims for optimal immobilization of purified plant α-amylase (purified from seeds of *Triticum aestivum*) through covalent attachment to functionalized graphene using RSM. Various factors (amount of graphene, cysteamine, glutaraldehyde and amount of protein) for optimal immobilization were evaluated and quantified using statistical approach of RSM. Subsequently, enzyme kinetics has been also studied to obtain best catalytic performance and enhanced reusability with greater storage ability. Also, the attachment was characterized by Scanning and Transmission Electron Microscopy (SEM, TEM), followed by Fourier transform infrared (FTIR) analysis.

2. Materials and methods

Dry seeds of *T. aestivum* were purchased from a local market. The chemicals for buffers preparation were of analytical or electrophoretic grade from Merck Eurolab GmbH Damstadt, Germany. All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Milli Q (Millipore, Bedford, MA) water with a resistance of higher than 18 MΩ was used all throughout the experiments.

α-Amylase was purified from 36 h germinated seeds of *T. aestivum* with a combination of salt fractionation and chromatographic techniques to electrophoretic homogeneity as ascertained by SDS-PAGE and MALDI-TOF [22].

2.1. Protein assay

Protein estimation was done by the Folin's Lowry method [23], using crystalline bovine serum albumin as standard protein. The immobilized protein was determined by subtracting the protein estimated in supernatant after immobilization from the total amount of protein used for immobilization.

2.2. Enzyme assays

The activity was measured using Fuwa's method [24].

2.2.1.Soluble

Starch (0.5 mL, 1%; w/v) with 0.3 mL sodium acetate buffer (100 mM, pH 5.0) and 0.1 mL double distilled water was incubated at 68 °C for 10 min. It was then followed by addition of 0.1 mL of suitably diluted enzyme and processed for 5 min. Reaction was stopped by denaturing the enzyme, using 0.5 mL 1 N HCl followed by cooling on ice. To 0.2 mL reaction mixture, 0.1 mL 1 N HCl and 0.1 mL iodine solutions were added. Final volume was made to 15 mL and absorbance was observed at 610 nm. One unit of α-amylase was defined as amount of enzyme, which caused a decrease of absorbance, by 0.05 in starch iodine color under assay conditions.

2.2.2. Immobilized

Starch (0.5 mL, 1%; w/v was incubated with 0.4 mL sodium acetate buffer (100 mM, pH 5.0) and 0.1 mL double distilled water, at 68 °C for 10 min. It was then transferred to an Eppendorf tube containing the immobilized amylase and processed the reaction for 5 min at 68 °C. The Eppendorf tube containing reaction mixture was then centrifuged for 2 min at 4 °C in order to easily separate the supernatant. Supernatant was further transferred to a test tube containing 0.5 mL 1 N HCl. To 0.2 mL reaction mixture, added 0.1 mL 1 N HCl and 0.1 mL iodine solution and made the final volume to 15 mL. Absorbance was read at 610 nm. One unit of α-amylase was defined as amount of enzyme, which caused a decrease of absorbance by 0.05 in starch iodine color under assay conditions.

2.3. Immobilization efficiency

The efficiency of enzyme immobilization was calculated using following relation:

$$\text{Immobilization efficiency (\%)} = \frac{\text{Specific activity of immobilized enzyme}}{\text{Specific activity of soluble enzyme}} \times 100$$

2.4. Functionalized graphene sheet preparation and characterization

Functionalized graphene was prepared by thermal exfoliation of graphite oxide following the protocol given by Staudenmaier [25,26]. Graphite powder (< 50 μm, 1 g) was reacted with strong oxidizing solution of conc. H_2SO_4, HNO_3 and $KClO_3$ at room temperature with constant stirring. Thereafter, graphite oxide solution was washed consecutively with distilled water and 10% HCl solution to remove sulfate and other ionic impurities followed by drying under vacuum at 80 °C. Next, it was thermally exfoliated to synthesize graphene by rapid heating under an Ar atmosphere. The sample was flushed with Ar gas for 15 min, and the quartz tube was quickly inserted into a tube furnace pre-heated to 1050 °C and held in the furnace for 30 s followed by cooling it down to room temperature under Ar gas flow. Thermally exfoliated functionalized graphene hence prepared was light weight shiny black powder rather than brownish graphite oxide. Functionalized graphene was suspended in Milli Q water followed by sonication at room temperature for 10 min and left undisturbed for 30 min so that the larger non-exfoliated flakes settled down. Slowly, the suspended functionalized graphene was collected leaving the larger flakes undisturbed.

Table 1
Box–Behnken experimental design for independent variables and their corresponding observed and predicted values of response (% immobilization).

Run	Cysteamine (mM)	Glutaraldehyde (% v/v)	Functionalized graphene (μg)	Enzyme (μg)	Immobilization	
					Actual	Predicted
1	5	1	1000	400	55.16	53.29
2	15	1	1000	400	40.03	40.56
3	5	4	1000	400	65.73	63.25
4	15	4	1000	400	52.95	49.87
5	10	2.5	500	200	33.46	33.22
6	10	2.5	1500	200	60.68	58.35
7	10	2.5	500	600	79.86	80.24
8	10	2.5	1500	600	60.04	58.33
9	5	2.5	1000	200	56.24	56.33
10	15	2.5	1000	200	36.45	35.54
11	5	2.5	1000	600	71.66	72.10
12	15	2.5	1000	600	69.35	66.79
13	10	1	500	400	45.93	45.12
14	10	4	500	400	56.92	56.46
15	10	1	1500	400	48.44	48.43
16	10	4	1500	400	56.02	56.36
17	5	2.5	500	400	67.73	70.86
18	15	2.5	500	400	42.86	42.72
19	5	2.5	1500	400	54.82	57.38
20	15	2.5	1500	400	56.40	57.55
21	10	1	1000	200	34.17	35.74
22	10	4	1000	200	44.16	45.98
23	10	1	1000	600	59.25	59.85
24	10	4	1000	600	67.14	68.88
25	10	2.5	1000	400	76.91	75.24
26	10	2.5	1000	400	75.96	75.24
27	10	2.5	1000	400	73.62	75.24
28	10	2.5	1000	400	72.45	75.24
29	10	2.5	1000	400	78.24	75.24

Table 2
Analysis of variance (ANOVA) for generated response surface model.

Source	Sum of squares	Df	Mean square	*F*- value	*p*-value Prob > *F*	
Model	5175.88	14	369.71	94.16	< 0.0001	Significant
***A*—Cysteamine**	510.91	1	510.91	130.13	< 0.0001	
***B*—Glutaraldehyde**	278.69	1	278.69	70.98	< 0.0001	
***C*—Graphene**	7.74	1	7.74	1.97	0.1820	
***D*—Enzyme**	1657.46	1	1657.46	422.15	< 0.0001	
AB	0.11	1	0.11	0.027	0.8721	
AC	174.90	1	174.90	44.55	< 0.0001	
AD	59.91	1	59.91	15.26	0.0016	
BC	2.91	1	2.91	0.74	0.4040	
BD	0.37	1	0.37	0.093	0.7646	
CD	553.19	1	553.19	140.89	< 0.0001	
A^2	550.17	1	550.17	140.13	< 0.0001	
B^2	1323.35	1	1323.35	337.05	< 0.0001	
C^2	568.54	1	568.54	144.80	< 0.0001	
D^2	451.00	1	451.00	114.87	< 0.0001	
Residual	54.97	14	3.93			
Lack of fit	37.25	10	3.73	0.84	0.6267	Not significant
Pure error	17.72	4	4.43			
Cor. total	5230.85	28				

$R^2 = 0.9895$; R^2 (adjusted) = 0.9790; Df = Degree of freedom.

The biochemical coupling of enzyme to the functionalized graphene was assisted by a spacer arm (cysteamine) and a cross-linker (glutaraldehyde). The functionalized graphene suspension, prepared in Tris buffer (50 mM, pH 8.0) to reach to a final concentration of 1 mg/mL, was divided into 29 aliquots according to the design of experiment (Table 1). The graphene aliquots were equilibrated in the same buffer (1 mL reaction volume) for overnight followed by thorough rinsing with the same buffer. Next, it was treated with cysteamine, prepared in the same buffer (1 mL reaction volume) and kept under dark condition for 4 h at room temperature. After cysteamine treatment it was washed with the same buffer followed by glutaraldehyde treatment (1 mL reaction volume) under similar conditions for 4 h. Washing was done for one more time with Tris buffer followed by overnight incubation with the enzyme under dark conditions at 4 °C. The immobilized enzyme was washed thoroughly with chilled buffer (50 mM Tris, pH 8.0) and immobilization was checked with activity assays under standard conditions. Each steps of immobilization was followed by thorough washing with chilled buffer (1 mL, washing two times) and centrifugation at 12,000 rpm for 5 min to settle down the graphene aliquots. Table 1 shows the list of independent variables according to the experimental design and their

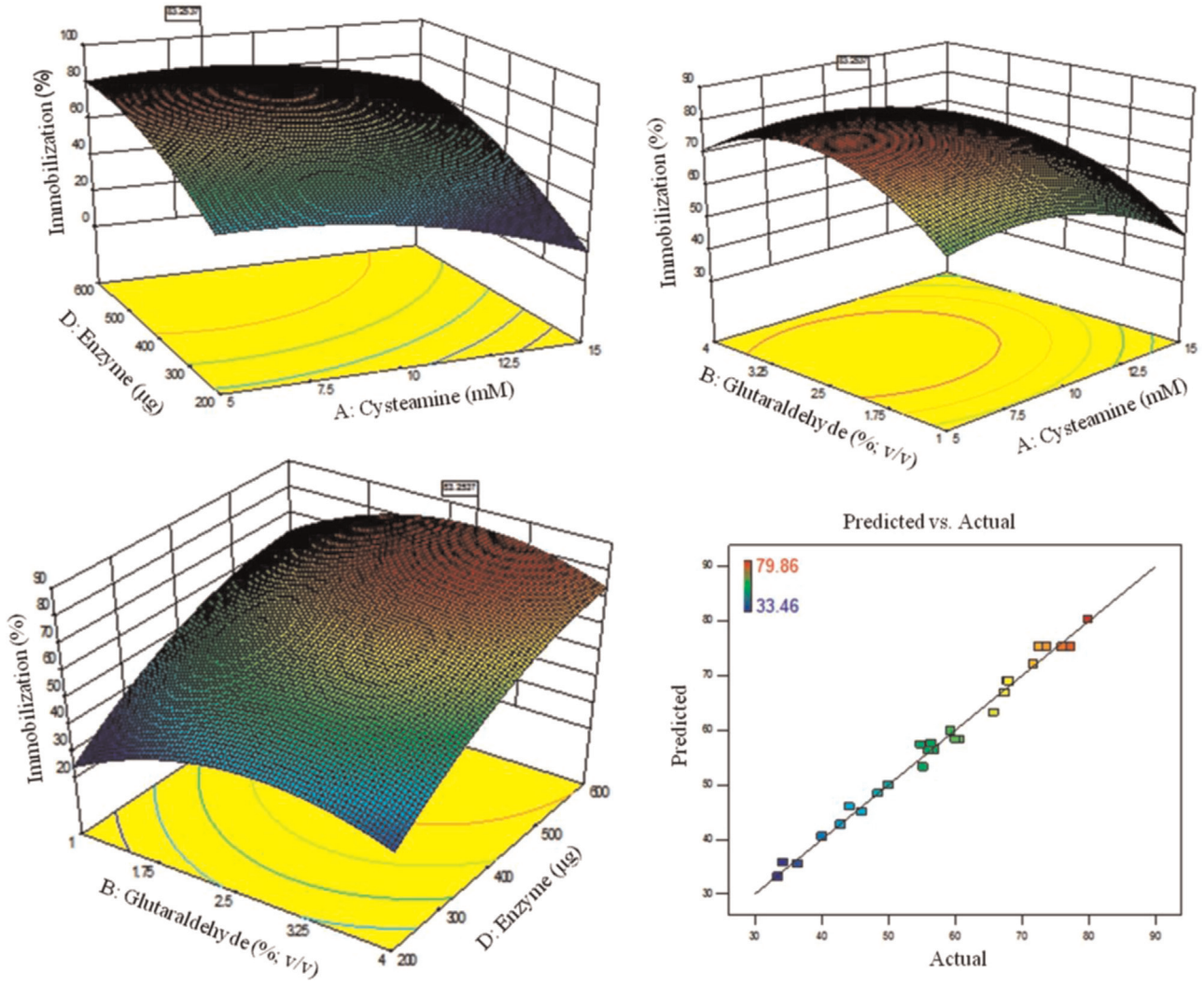

Fig. 1. Represents response surface plots representing interaction of two variables with their effect on percentage immobilization and predicted versus experimental responses of interacting variables.

corresponding response.

Functionalized graphene sheets (both native and coupled) were characterized using transmission electron microscope (TEM; Technai 20 G2, 200 kV), scanning electron microscope (SEM; Philips: XL 20) and Fourier transform infrared spectroscopy (Perkin Elmer Spectrum 100 instrument). For TEM studies, a drop of sample was placed on electron microscope 200 mesh copper grid and allowed to evaporate the water to complete dryness of the sample, followed by loading into the machine. For SEM studies, samples were sprinkled on the stub having layer of silver glue for striking the particles. The fine structural details were obtained using secondary electron imaging mode. FTIR was performed in the range of 400–4000 cm^{-1} wave numbers, with 100 scans of each sample to obtain a good signal to noise ratio.

2.5. Experimental setup and statistical analysis

Before attempting multivariate statistical design, preliminary experiments were carried out to screen the significant factors and determine their initial values which affected the quality of the derived outcome (data not shown). On the basis of these experiments, a three level study of the significant parameters and their interactions (amount of functionalized graphene, amount of enzyme and concentration of cysteamine and glutaraldehyde), were analyzed and optimized using Box–Behnken design. This response surface statistical experimental design consisted of 29 trials with independent variables being studied at 3 different levels. The variables and their levels selected for obtaining the immobilization of α-amylase onto functionalized graphene were: amount of functionalized graphene (500, 1000 and 1500 μg), cysteamine concentration (5.0, 10.0 and 15.0 mM), glutaraldehyde concentration (1.0%, 2.5% and 4.0%) and amount of protein (200, 400 and 600 μg). All experiments were done in duplicate and the average of immobilization obtained was taken as the dependent variable/response (Y). 'Design Expert' software (Version 8.0, Stat-Ease Inc., Minneapolis, MN) was used for the experimental design, data analysis and quadratic model building [27]. The mathematical relationship, involving a second-order polynomial model, used to estimate the effects of various factors on a response is given by

$$Y_i = \beta_0 + \sum^{\beta i} X_i + \sum^{\beta ii} X_i^2 + \sum^{\beta ij} X_i X_j$$

where Y_i is the predicted response X_iX_j are input variable which influence the response variable Y_i; β_0 is the offset term; β_i is the ith linear coefficient; β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient. Statistical analysis of variance (ANOVA) which included lack of fit, Fischer's F-test, its associated probability $p(F)$ and correlation coefficient R to measure the goodness of fit of the quadratic model. Response surface and contour plots were generated to understand the interaction of different

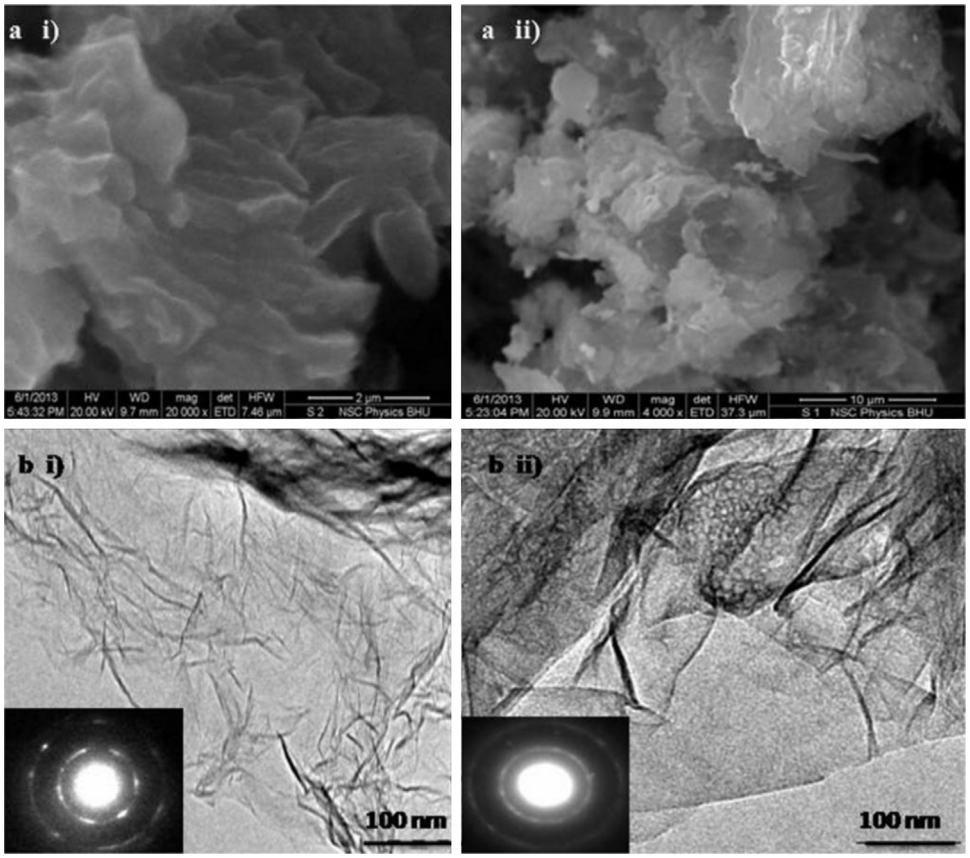

Fig. 2. (A) SEM images of functionalized and coupled graphene. (B) TEM images of functionalized and coupled graphene with inset showing the selected area electron diffraction pattern.

variables. For the present study, a total of 29 tests were performed to estimate the coefficients. Thereby, the validity of generated mathematical model was checked by conducting experiments at given optimal conditions.

2.6. Steady state kinetics

In each case, control experiment using soluble enzyme was carried out.

The optimum pH for immobilized wheat α-amylase was studied in the pH range 3.0–9.0 (citrate buffer: 3.0–3.5, acetate buffer: 3.6–5.5, sodium phosphate: 6.0–7.0, Tris buffer: 7.5–9.0) at 68 °C. In all cases, 100 mM buffers were used and activity was observed as stated in the method. The effect of temperature on coupled enzyme was determined in temperature range, 35–85 ± 1 °C, using a multitemp water bath (Pharmacia, Uppsala, Sweden). For thermal inactivation studies, the soluble and immobilized enzymes (in aliquots) were incubated at 67 °C in water bath for different time intervals, followed by residual activity assay under standard conditions. The enzyme activity as a function of substrate concentration was measured for both soluble and coupled enzyme by varying substrate concentration in standard assay procedures. Data thus obtained was used for calculation of K_m (Michaelis–Menton constant) using Lineweaver–Burk plot. The aliquots of functionalized graphene coupled with enzyme conjugate were stored under wet conditions at 4 °C and the residual activity was checked at different time intervals using standard assay conditions. For reusability assessment, stored immobilized enzyme was repeatedly used 10 times and the residual activity was checked. After assay, the immobilized preparation was washed with 50 mM Tris buffer, pH 8.0; to remove any attached substrate. Furthermore, the immobilized α-amylase, which showed better stability, was reused for prolonged periods.

3. Results and discussion

3.1. Process optimization

As suitable immobilization conditions are critical to maximize the multipoint covalent attachment, statistical optimization by RSM offers the opportunity to find out the optimal levels of process variables under any given condition by establishing the relationship between factors and the predicted responses [28]. In the preliminary work, operational ranges of variables having favorable effect on response were selected. Subsequently RSM was applied using Box–Behnken design within these ranges of factors to obtain maximum response (Table 1). Based on these experiments, a mathematical model was developed to obtain the maximum immobilization response within the set range of variables and following points were determined: Cysteamine: 8.19 mM; Glutaraldehyde: 2.88% (v/v); Functionalized graphene: 615.36 μg; Enzyme: 553.09 μg; Immobilization: 83.25%. Supplementary experiments to check the validity of these predicted values, generated from the second order quadratic equation were performed and about 85.16% immobilization was achieved, which was in agreement with the predicted value.

Approval of this model was validated with help of ANOVA (Table 2). The analysis of variance of quadratic regression model demonstrated that this model is highly significant with *F*-value of 94.16 and there is only 0.01% chance that this value could occur due to noise. The “Adj *R*-squared” of 0.9790 is in agreement with “Pre *R*-squared” of 0.9537. The “Lack of Fit *F*-value” of 0.84 implied that it is not significant relative to the pure error. Adequate

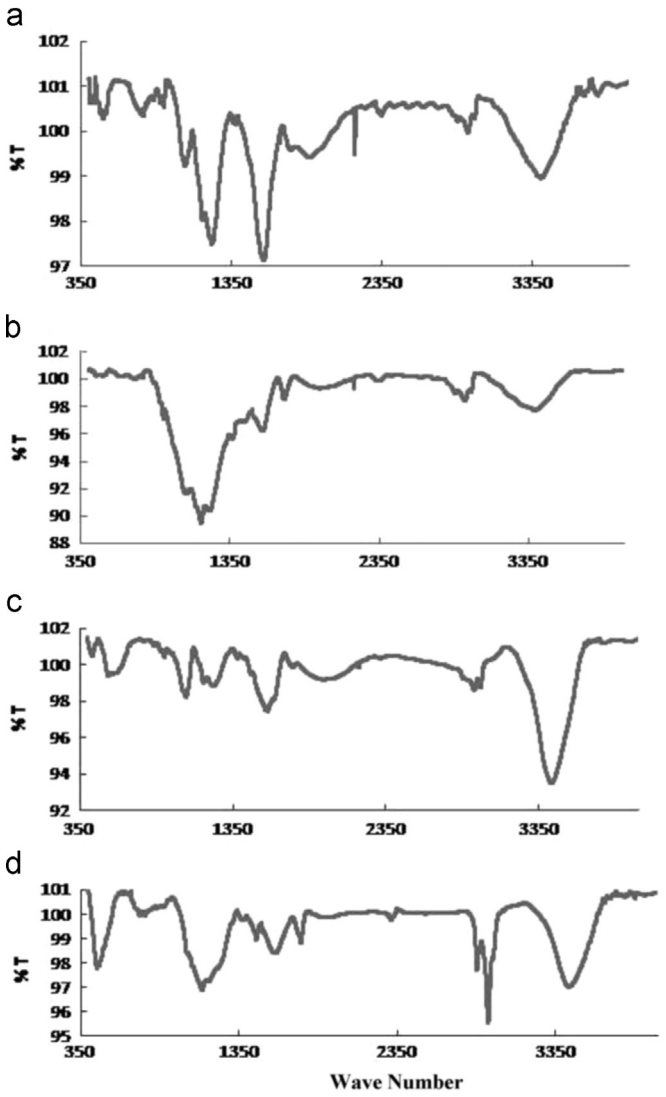

Fig. 3. FTIR spectra of immobilized, glutaraldehyde treated, cysteamine treated and functionalized graphene (A–D), respectively.

precision measures the signal to noise ratio and a value greater than 4 indicates adequacy.

Based on these results, the model can be utilized to generate response surfaces and contour curves for the analysis of the variable effects on immobilization (Fig. 1). The response surface and contour curves were obtained using following equation:

$$\begin{aligned}\text{Immobilization efficiency} = &-101.67644 + 1.92390 \times A \\ &+ 19.73576 \times B + 0.09993 \times C \\ &+ 0.30695 \times D \\ &-0.02166 \times A \times B + 0.002645 \times A \times C \\ &+ 0.00387 \times A \times D - 0.00113 \times B \times C \\ &-0.00101 \times B \times D - 0.00011 \times C \times D \\ &-0.36839 \times A^2 - 02 - 0.34819 \times B^2 - 02 \\ &-0.00003 \times C^2 - 02 - 0.00021 \times D^2\end{aligned}$$

where A is the cysteamine concentration (mM); B is the glutaraldehyde concentration (%; v/v); C is the amount of functionalized graphene (mg) and D represents amount of enzyme (mg).

The results are indicative for good precision and reliability for the experiments carried out for optimal immobilization.

3.2. Characterization

Functionalized graphene and changes in its property upon treatment with cysteamine, glutaraldehyde and enzyme was characterized using SEM and TEM, followed by FTIR spectroscopy. Modulation in surface topography of functionalized graphene, before and after immobilization is quite evident (Fig. 2). Upon immobilization, transparent sheets of functionalized graphene are occupied by islands of immobilized enzyme. Attachment of enzyme also leads to an alteration in characteristic selection area electron diffraction (SAD) pattern of functionalized graphene. SAD of the microstructure (Fig. 2b) clearly shows the polycrystalline ring of the graphene sheet (inset) in both the images (Fig. 2b(i) and b(ii)). However diffusion spot is also observed in the SAD pattern of the enzyme immobilized graphene sheet (Fig. 2b(ii)) which clearly indicates that some interaction has taken place between functionalized graphene and enzyme, which appears as diffused ring in the SAD.

FTIR spectra for native, cysteamine treated, glutaraldehyde treated and enzyme immobilized graphene sheets were taken in order to get an insight regarding interaction between functionalized graphene and enzyme molecules (Fig. 3). The spectra showed carbonyl stretch (C=O) of carboxylic group at 1740 cm^{-1}. Peaks at 1116.05 cm^{-1} and 1163.5 cm^{-1} corresponds to C–OH (hydroxyl) and C–O (epoxy) stretching, respectively; thereby confirming the presence of oxygen containing functional groups e.g. C=O, C–OH and C–O on functionalized graphene sheets. Thereafter, it is attached to cysteamine, as evident from peak at 1160 cm^{-1} showing C=S stretching corresponding to thiocarbonyl group hence indicating that cysteamine functionalizes graphene sheets through –SH group while other end containing $–NH_2$ group remains available for glutaraldehyde attachment. Next, the functionalized graphene sheets were treated with glutaraldehyde where one arm of glutaraldehyde binds to the $–NH_2$ group of cysteamine through –CHO group while the other arm remains free for attachment with enzyme *via* lysine residues. This interaction is confirmed by the observed peak at 2172 cm^{-1} for N=C=O stretch. Final step involves attachment of enzyme to the free arm of glutaraldehyde via lysine amino group. Resultant FTIR spectra showed many prominent peaks with bands at 1671 cm^{-1} represent carbonyl amide I bonds whereas bands at 1562 cm^{-1} represents amide II bonds. As the enzyme is glycoproteinaceous in nature, peak at 1045.2 cm^{-1} represents C–O–C of glycosylated residues which further confirmed the immobilization of wheat α-amylase onto functionalized graphene.

3.3. Steady state kinetics

Upon enzyme immobilization, kinetic parameters often experience an alteration in optimal properties over their soluble form as their behavior can be modified by its immediate microenvironment. The pH optima of immobilized form did not show any change in comparison to soluble enzyme (pH optima, 5.0) (Fig. 4a). However, immobilized wheat α-amylase had broad pH profile in the range of 3.0–9.0 indicating that the effective rate of the enzyme becomes less sensitive to pH changes [29]. Enzyme did not exhibit any change in optimum temperature after immobilization (67 °C) similar to soluble enzyme, when assayed with starch under standard condition (Fig. 4b). However, thermal stability of immobilized enzyme was significantly improved. Thermal denaturation kinetics, studied for both soluble and immobilized enzyme at 67 °C showed that after 120 min of incubation, the immobilized enzyme had 43% residual activity whereas soluble enzyme had 19% residual activity (Fig. 4c). Enzyme immobilization using glutaraldehyde provides multipoint attachment which contributes to strengthen it's proper backbone structure in three

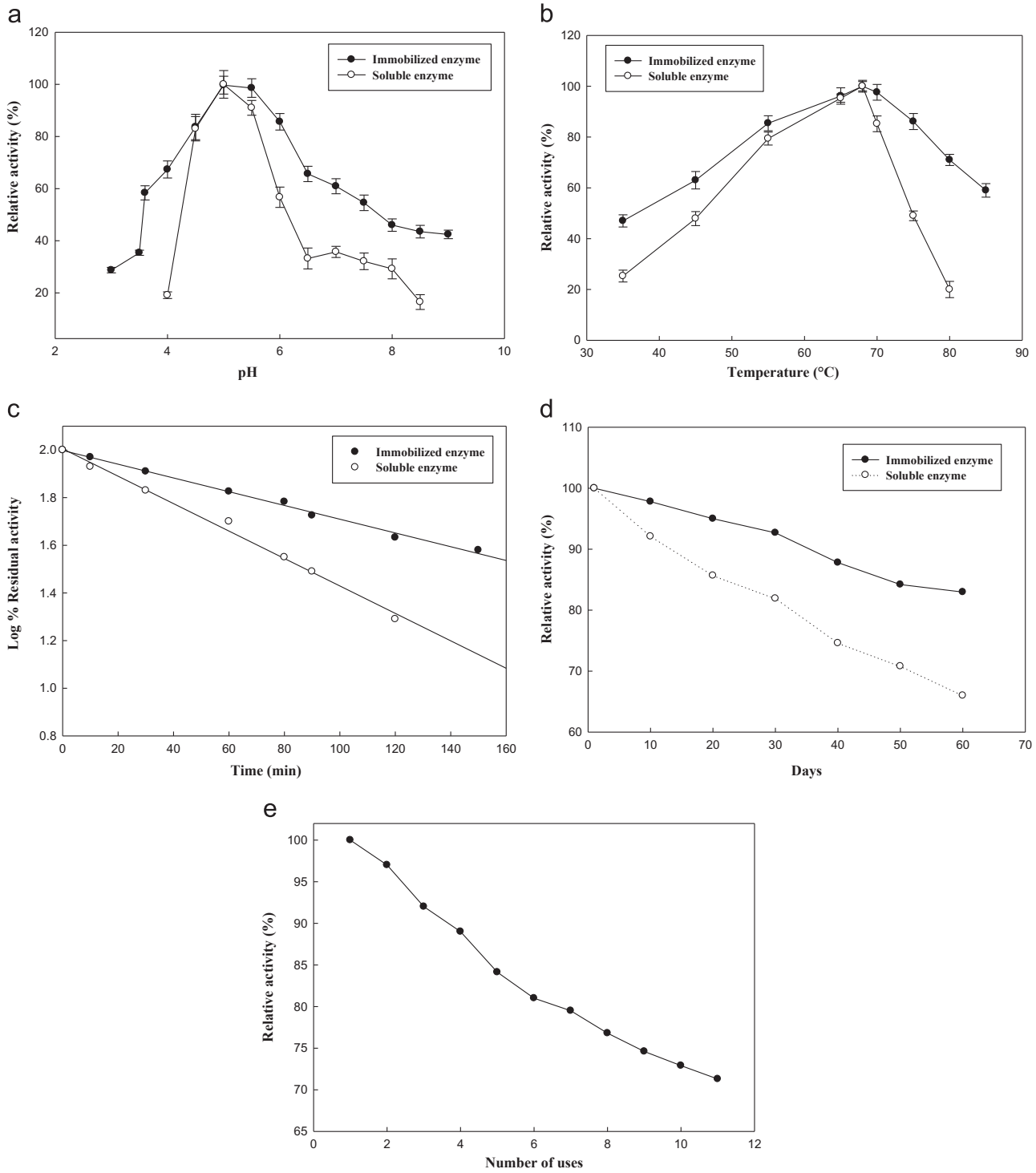

Fig. 4. Effect of (a) pH (citrate buffer: 3.0–3.5, acetate buffer: 3.6–5.5, sodium phosphate: 6.0–7.0, Tris buffer: 7.5–9.0) at 68 °C (b) temperature (35–85 ± 1 °C) on soluble and immobilized enzyme using 1% starch. (c) Thermal inactivation kinetics for soluble and immobilized enzyme at 67 °C for different time intervals, followed by residual activity assay under standard conditions. (d) Storage stability of soluble and immobilized enzyme (0.1 M acetate buffer, pH 5.0) at 4 °C under wet condition and (e) represents reusability (10 cycles) of immobilized α-amylase.

dimensions thus rendering it unaccessible at high temperatures. A minor decrease in the K_m value was observed upon immobilization of enzyme as it changed from 1.56 mg/mL to 1.32 mg/mL suggesting slight change in microenvironment resulting from the enzymatic hydrolysis of substrate whereas in case of Amberlite immobilized α-amylase, K_m was 2.5 mg/mL resulting from mass transfer limitations which is minimized in case of graphene nanosheeth [3].

3.4. Storage stability and reusability

Enzymes are very sensitive biocatalysts against environmental conditions and may lose their activities quite easily. Hence, for any immobilized enzyme to gain grounds in industrial sector, storage stability and reusability are often looked upon as influential characteristics controlling the economics of any bioprocess. Storage stability is an intrinsic property of the enzyme and can be

greatly improved by cross-linking it to some suitable matrix [30]. The residual activity, for free amylase and immobilized enzyme after 60 days of storage under wet condition at 4 °C was 65% and 83%, respectively (Fig. 4d) which was found to be 40% after 100 days of storage for chitosan immobilized-α-amylase [3].

The reusability of immobilized enzyme is a key quality for the economic feasibility of bioprocess fixed in immobilized enzyme system. After 10 uses, the residual activity of immobilized wheat α-amylase was 73% (Fig. 4e) whereas that of chitosan immobilized α-amylase was 58% [3]. With repeated use of immobilized enzyme, leaching of enzyme occurs due to the weakening of binding strength between the nanosupport and the immobilized enzyme. Frequent encountering of substrate into the active site causes its distortion, thus reduces its catalytic efficiency either partially or fully. In conclusion, for competent bioconjugate assembly, statistically designed experimentation through Box–Behnken design was utilized to derive efficacy for the immobilization process. For successful industrial endeavor, enzyme needs to preserve its functional capacity under conditions of extreme pH and temperature. This nanosupport based bioprocess system is benefitted with improved thermal and storage stability, increase in affinity towards substrate and imparts operative value for industrial sector with broad pH range. With aforementioned features, graphene coupled wheat α-amylase promises adherence and endurance for continuous biotechnological processes in starch based industrial sector and as biosensors for the determination of starch content.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2015.07.002.

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