Thermo-responsive super porous p(NIPAM) cryogels affords enhanced thermal stability and activity for a-Glucosidase enzyme by entrapping in situ

Sahin Demirci[1] and Nurettin Sahiner[2]

[1]Canakkale Onsekiz Mart Universitesi Fen Edebiyat Fakultesi
[2]University of South Florida College of Medicine

January 30, 2021

Abstract

The concept of using a thermo-responsive p(NIPAM) polymer matrix for enzyme immobilization with lower critical solution temperature (LCST) value is rationalized by availability of the compartmental milieu to enzymes to operate within super porous 3-D matrix with special environmental conditions. Therefore, the enzyme immobilization within a support material will be carried out under the storage conditions of enzymes, generally ~-20 oC to afford unnecessarily loss of enzyme functionality in comparison to the other enzyme entrapment methods. Thus, here a-Glucosidase as a model enzyme was entrapped within thermo-responsive super porous p(NIPAM) cryogels (a-Glu@p(NIPAM) during the synthesis that uses cryogenic condition, ~-20 oC. The LSCT value for the prepared p(NIPAM) based cryogels were determined as 34.6±1.2 oC. The immobilization yield, immobilization efficiency, and activity recovery% values were calculated as 89.4±3.1, 66.2±3.3, and 74.0±3.3%, respectively at pH 6.8 and 37 oC for a-Glu@p(NIPAM) cryogel system. Interestingly, the optimum working conditions were achieved as 25 oC and pH 6.8 with higher activity, 98.4±0.2% for the prepared a-Glu@p(NIPAM) cryogel system. The operational and storage stability studies revealed that the prepared a-Glu@p(NIPAM) cryogel system possessed much better operational and storage stability than free a-Glu enzyme e.g., more than 50% activity after 10th usage and 10-day room temperature storage time. Moreover, the kinetic parameters such as Km and Vmax of free-Glu enzyme and a-Glu@p(NIPAM) cryogel system were calculated by non-linear Michaelis-Menten equation.

1. Introduction

Enzymes are biocompatible and biodegradable natural catalysts with high selectivity operating in mild conditions e.g., physiological pH and temperatures, atmospheric pressure and in aqueous environments (Hanefeld et al., 2009; Ho, 2012; Zhu et al., 2012). Although enzymes have the advantages of operating quite specifically and effectively, they have drawbacks such as expensive cost of effective enzymatic processes e.g., low stability for long-term use due to their solubility, denaturing feature at high temperature because of their protein structure, and demonstrating low activity at high and low temperatures and pH values (Forsyth and Patwardhan, 2013). Various techniques to improve enzyme stability employing various methods and engineering such as enzyme modification and enzyme immobilization have been proposed (Ding et al., 2016; Yang et al., 2017). Among these approaches, enzyme immobilization holds out the most effective and widely researched method due to the possibility of recycling, continuous operation, ease of product purification (Yang et al., 2017). The term immobilization implies to the attachment of biologically active molecules to a support (carrier) material to enable no or minimal loss in their biological activity, and this technique commonly used for enzymes to increase stability and maintain activities in harsher conditions i.e., low/high temperature and pH (Hamerska-Dudra et al., 2007). Enzyme immobilization facilitates the purification of the reaction system and the recovery of the enzyme as well as tolerating the repeated or continuous use

of enzymes. So, as a result of enzyme immobilization, some changes may occur in the enzymatic activity, optimum operating temperature and pH values, affinity for the substrate, and the stability of the enzymes in comparison to the free enzymes traits. And, all these factors depend on the type of enzymes, the support material that the enzyme is immobilized, and the immobilization conditions (Huang et al., 2018; Liu and Chen, 2016; Petrosino et al., 2019; Wang et al., 2020). The systems created with enzyme immobilization has combined specific physical, chemical, biochemical, mechanical and kinetic properties of both the enzyme and the support material (Homaei et al., 2013). It has been reported that various matrices such as synthetic, organic and biological polymers can be used as support materials for enzyme immobilization (Aktaş Uygun et al., 2015; Lin et al., 2021; Mondal et al., 2017; Sheldon, 2007). Materials recommended as support in many instances are not suitable for industrial processing procedures due to their low mechanical strength, unsuitable chemical functionality and in appropriate physical conditions (Sakaguchi et al., 2005).

3-Dimensional (3D) polymeric matrices with nano-, micro- and super pore structure are desirable in many biomedical applications including catalysis, drug delivery, and protection of proteins, enzymes, DNA, and other active bio-macromolecules (Wang et al., 2015). Cryogels with interconnected super-porous network structures differ from their conventional hydrogel counterparts as they are also cross-linked network structures but prepared via cryogelation of appropriate monomers or (bio)polymer precursors at subzero temperature (Lozinsky, 2002). The most important properties of cryogels stem from their interconnected super-porous structures empowering fast response, high elasticity and mechanical endurance (Henderson et al., 2013; Lozinsky et al., 2003). Another important features of cryogels is the use of water in ice form in order to form interconnected mostly continuous pores that make cryogels feasible in biological and biomedical applications (Henderson et al., 2013). Many different cryogel structures have been reported as support material for enzyme immobilization by using various methods (Armutcu et al., 2020; Aslanli et al., 2019; Chambre et al., 2020; Yavaşer and Karagözler, 2021). In reported studies, adsorption and covalent binding techniques of enzymes as immobilization to cryogels stand out as the mostly widely employed methods (Ingavle et al., 2015; Jahangiri et al., 2014; Laochai et al., 2016). Smart polymers also considered high potential supports in enzyme immobilization as these polymers capable of changing their physical and chemical structures with the changes in external stimuli e.g., temperature, pH, magnetic/electric field, solvent and so on that all have an effect on enzyme stability and activity (Ding et al., 2016; Hamerska-Dudra et al., 2007; Homaei et al., 2013; Wang et al., 2015; Yang et al., 2017).

The use of gels from smart polymers or other structures modified with smart polymers are employed as support in enzyme immobilization (Ding et al., 2016; Hamerska-Dudra et al., 2007; Homaei et al., 2013; Wang et al., 2015; Yang et al., 2017). The NIPAM modified magnetic Fe_3O_4 and Fe_3O_4@SiO_2 particles were used as a carrier for lipase enzyme immobilization via adsorption method (Wang et al., 2015). The trypsin and glucoamylase enzymes are immobilized within co-polymeric hydrogels of NIPAM-HEMA, and NIPAM-GMA via covalent bonding method (Hamerska-Dudra et al., 2007). The p(NIPAM) hydrogels were used as support materials for pepsin enzyme immobilization via covalent bonding method (Cirillo et al., 2014). In another study, the urease enzyme was immobilized within p(NIPAM) hydrogels and cryogels by UV irradiation method, and the comparison of activity% and reusability properties of prepared systems to free urease enzyme were investigated (Petrov et al., 2011).

Differently from literature, in this study, superporous p(NIPAM) cryogels as temperature sensitive matrix were synthesized under cryogenic conditions (-20 °C) via simultaneous solution polymerization and crosslinking in the presence of various amounts of a-Glu enzyme to entrap within 3-D p(NIPAM) cryogel network. The immobilization parameters such as immobilization yield%, efficiency% and activity recovery% were calculated. The activity% of a-Glu@p(NIPAM) cryogel systems was measured and compared with equivalent amount of free a-Glu enzymes. The activity% of immobilized and free enzyme were compared at 20-60 °C temperature and pH 4-9 ranges. The reusability and stability of the immobilized enzyme at different temperature as storage conditions were studied. Additionally, K_m and $V_{\max}$ kinetic parameters were calculated and compared for free and immobilized enzyme.

2. Experimental

2.1 Materials

N-isopropylacrylamide (98%, Sigma Aldrich) as the monomer, N,N'-methylene bis-acrylamide (98%, Sigma Aldrich) as the crosslinker, N,N,N,N-tetramethyethylenediamine (98%, Merck) as the catalysts, and potassium persulfate (KPS, 98%, Sigma Aldrich) as the initiator were used in the synthesis of supermacroporous p(NIPAM) cryogels. The enzyme, a-Glucosidase from *Saccharomyces cerevisiae* (a-Glu, 100 Unit/mg, Sigma Aldrich) was used to in situ entrap into p(NIPAM) cryogel and p-nitrophenyl-a-D-glucopyranoside (4-NPG, 99%, Acros) were used as substrate in the enzymatic reactions and Reduced L-Glutathione (98%, Aldrich) was used as co-factor. Sodium carbonate (Na_2CO_3, 99%, Merck) was used for the termination of free enzyme reactions. Potassium phosphate monobasic (KH_2PO_4, 98-100.5%, Sigma Aldrich), sodium hydroxide (NaOH, 99.9%, VWR chemicals), and hydrochloric acid (HCl, 36.5%, Sigma Aldrich) were used for the preparation of appropriate buffer solutions. Rosmarinic acid (RA, 96 %, Aldrich) was used as inhibitor. Double distilled (DD) water used in the preparation of aqueous solutions.

2.2 Synthesis of p(NIPAM) and a-Glu@p(NIPAM) cryogels

The super porous p(NIPAM) and a-Glu@p(NIPAM) cryogels were synthesized at -20 °C, cryogenic conditions by following earlier reported study with some modifications (Sahiner, 2018) using the chemicals summarized in **Table 1S** . In brief, 7.5 mmol of NIPAM monomer was dissolved in 12 mL of DD water and then combined with 1.1 mmol MBA as crosslinker and 100 µL of TEMED as accelerator were consequently added into monomer solution and dissolved by stirring at 500 rpm mixing rate for 10 min. Then, 1 mL of aqueous KPS solution that accounts 1 mole % of NIPAM monomer as initiator was freshly prepared in a separate vial. Next, both monomer and initiator solutions were placed into deep freezer (-20 °C) for 5 min to chill. Subsequently, these cooled solutions (monomer and initiator) were mixed quickly vortexed for 30 sec, and transfer into plastic straws (8 mm of diameter) quickly and placed into deep freezer at -20°C. The concurrent polymerization and crosslinking at cryogenic conditions were continued for 20 h. Finally, the prepared p(NIPAM) cryogels were cut in similar shapes of about 0.6 mm in diameter, and 0.8 mm in height, and washed in excess amount of water twice, and dried in freeze-dryer (Christ, Alpha 2-4 LSC) at -86°C. The washed, dried p(NIPAM) cryogels were stored in zip-lock bags for characterization and further use.

The enzyme entrapped p(NIPAM) cryogels (a-Glu@p(NIPAM)) were also prepared by using the method as described above. In short, 2, 4, and 8 mL of 12 mL DD water were replaced with enzyme solution at 0.25 mg/mL a-Glu enzyme solutions that contain 50, 100 and 200 mg of enzymes. Similarly, the MBA, TEMED solutions were prepared as in the procedure as mentioned above was followed as in bare p(NIPAM) cryogel synthesis method. After 20 h simultaneous polymerization and crosslinking at cryogenic conditions, the prepared 2 ,4, and 8 mL a-Glu enzyme solutions containing cryogels as a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 were also cut in similar dimensions (˜0.6 mm in diameter and 0.8 mm in height) and washed with 20 mL of water for two times and dried in freeze-dryer. The washed and dried a-Glu@p(NIPAM) cryogel systems were stored in zip-lock bags at -20 °C for characterization and further usage.

2.3 Characterization

Scanning electron microscope (SEM) images of p(NIPAM) cryogels were taken (QUANTA 400F Field Emission SEM) after coating of a few nm of gold and via applying 20 kV of operational voltage.

The Fourier Transform Infrared (FT-IR, Spectrum, Perkin Elmer) spectrum of p(NIPAM) and a-Glu@p(NIPAM) cryogel systems were recorded between 4000-650 cm^{-1} wavelength by using attenuated total reflector (ATR) with 4 cm^{-1} resolution range and 4 repeating scan.

Thermal stability and degradation profiles of p(NIPAM) and a-Glu@p(NIPAM) cryogel systems were investigated by using thermogravimetric analyzer (TGA, Seiko, SII 6300, Exstar). Cryogels weighted in 3-5 mg was placed into TGA pan and the temperature was increased up to 750 °C at 200 mL/min Nitrogen gas flow at 10 °C/min heating.

The swelling%, porosity%, pore volume%, and gel yield% of p(NIPAM) and a-Glu@p(NIPAM) cryogel

systems were estimated from by calculation following the very well-known equations as given below (Plieva et al., 2005; Tripathi and Melo, 2015);

Swelling% = (m_s - m_d) / m_d x 100 (1)

Porosity% = (m_s - m_d) / (m_s - m_{sq}) x 100 (2)

Pore volume% = (m_s - m_d) / m_s x 100 (3)

cryogel yield% = (m_d / m_r) x 100 (4)

where the "m_s" designated for abbreviation of weight of swollen cryogels pieces in water, "m_d" is weight of the washed and freeze dried cryogel pieces, "m_{sq}" is the weight of squeezed cryogel pieces after swelled in water, and "m_r" is the weight of total amount of cryogel precursors, respectively.

2.4 The leakage studies and immobilization parameters

The a-Glu enzyme leakage studies from entrapped p(NIPAM) cryogels were calculated via enzymatic activity comparison of the same amounts of free enzymes with the used ones entrapped within cryogels. The washing solutions of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogels were tested in enzymatic assay and the activity was calculated by following equations in literature (Boudrant et al., 2020; Sheldon and van Pelt, 2013). The observed absorbance values at 400 nm were recorded to assess the enzyme leakage from a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems.

Some of the important immobilization parameters e.g., immobilization yield, immobilization efficiency, and activity recovery after reuse experiments were also calculated from enzymatic activity of washing solutions, free enzyme and a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems following the reported methods in literature (Boudrant et al., 2020; Sheldon and van Pelt, 2013).

2.5 Enzymatic activity

Enzymatic activity of free and entrapped a-Glu enzyme were calculated in accord with the established method (Demirci et al., 2020). In brief, the one piece of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems were placed in 5 mL of 67 mM PBS solutions at pH 6.8 with 20 μL of 0.2 mM reduced glutathione solution and incubated for 10 min at 37 . Then, 0.5 mL of 10 mM 4-NPG solution in water as substrate for enzyme was added in into the enzyme including buffer solutions and reacted for 20 min at 37 . DD water, 20 μL was added into the buffer solution instead of enzyme solution in the preparation of a control solution. The same procedure was also applied for control test. After 20 min, the enzymatic reaction was terminated by removing of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems from reaction buffer solutions. Finally, the absorbance values at 400 nm assigned to 4-NP as the product of enzymatic reaction was measured using UV-Vis spectrometer (T80+, PG Instruments).

The activity of free a-Glu enzyme was also calculated using the same method mentioned above. The equivalent amounts of a-Glu enzymes as in the entrapped a-Glu enzymes in p(NIPAM) cryogels were placed into 5 mL of pH 6.8 buffer solution at 0.25 mg/mL concentration with 20 μL of 0.2 mM reduced glutathione solution and incubated 10 min at 37°C, and after the addition 0.5 mL of substrate solution, the enzymatic reaction was performed at 37°C for 20 min. The termination of the reactions was done by adding 2 mL 100 mM Na_2CO_3solutions. Finally, the absorbance at 400 nm belonging to 4-Nitrophenol (4-NP) as the product of enzymatic reaction was measured by UV-Vis spectrometer.

The effect of the amounts of entrapped enzyme, the temperature, 20-60°C, and the solution pH, 4-9 range on activity% of free and entrapped a-Glu enzymes within p(NIPAM) cryogels were also investigated. The activity% of equivalent amount of free enzyme was assumed as 100%.

2.6 Kinetic studies

The kinetic parameters, K_m and V_{max} values for the enzymatic reactions were determined for free and entrapped a-Glu enzymes by using non-linear (Eq. 5) and linear (Eq. 6) Michaelis-Menten equations (also

called Lineweaver-Burk plot);

$V_o = (V_{max} \text{ x } [S])/ (K_m + [S])$ (5)

$1/V_o = K_m /(V_{max} \text{ x } [S]) + 1/V_{max}$ (6)

where, "V_o" is the initial rate (mM/min), "V_{max}" is the maximum velocity of reaction (mM/min), "[S]" is the substrate concentration (mM), and "K_m" is Michaelis-Menten constant described as (mM), respectively.

In the kinetic studies, the enzyme assay was carried out by adding of 0.5 mL of 2.5-40 mM substrate solution with a-Glu@p(NIPAM)-0.5 cryogel systems and with the system containing the equivalent amount of free enzyme in buffer solutions at pH 6.8. The kinetic parameters from non-linear Michaelis-Menten equation were estimated by using Excel-Solver (Hernández and Ruiz, 1998; Kemmer and Keller, 2010).

2.7 The operational and storage stability studies

The operational stability or reusability of free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel systems were investigated via the enzyme assay by using the same systems in ten times consecutive run in pH 6.8 buffer solution at 25 °C and 37 °C. The used a-Glu@p(NIPAM)-0.5 cryogel systems were washed with buffer solutions of pH 6.8 for 30 min after each use in the enzyme assay. The operational stability of a-Glu@p(NIPAM)-0.5 cryogel reactor systems at 25 and 37 °C were compared with each other. The decrease in the activity% of α-Glu entrapped cryogels was calculated assuming the activity% of the first usage of each set is 100% activity.

The storage stability of free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel systems were investigated by storing of both systems at -20, +4, and 25 °C for 10 days. The change in activity% of both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel systems were compared at every day of the stored enzymes with each other assuming the activity% in the first day for each set as 100% active.

2.8 Inhibition screening tests

To determine the inhibition profiles of a-Glu@p(NIPAM)-0.5 cryogel systems along with the equal amount of free enzyme containing solution, various concentrations of RA (Tadera et al., 2006) solutions, 1.8, 1.2, 0.9, 0.45, 0.22, and 0.11 mg/mL at pH 6.8 were used. In brief, the 1 mL of each RA solutions at 1.8, 1.2, 0.9, 0.45, 0.22, and 0.11 mg/mL were added into mixture of solution containing enzymes at pH 6.8. Then, the measured absorbance values at 400 nm were recorded for reaction involving the inhibitor, defined as A_{inh}, and the observed absorbance values for a-Glu@p(NIPAM)-0.5 cryogel system is defined as A_{test}, and were used in the calculation of inhibition%. The inhibition% of free and entrapped α-Glu enzymes was calculated using Eq. (7) (Zhu et al., 2014);

Inhibition % = $(A_{test} - A_{inh}) / A_{test}$ x100 (7)

3. Results and discussion

3.1 Synthesis and characterization of enzyme entrapped super porous p(NIPAM) cryogels

The inter connected pores of super porous cryogels deliver a intermediate for fast exchange of regents offering great potential in various applications (Henderson et al., 2013; Lozinsky, 2002; Lozinsky et al., 2003). By preparing cryogels from stimuli responsive sensitive smart polymers can confer materials with outstanding properties rendering many advantages in the biotechnological field. The use of stimuli responsive smart polymers for enzyme immobilization with various techniques in various morphology was reported by few groups (Ding et al., 2016; Hamerska-Dudra et al., 2007; Homaei et al., 2013; Wang et al., 2015; Yang et al., 2017). Our group reported the synthesis of super porous smart cryogels of p(NIPAM) for the separation for biomolecules (Sahiner, 2018). Therefore, the same route in p(NIPAM) cryogels preparation was used to formulate a-Glu enzyme entrapment with the exception of the replacement of water with varying volumes of enzyme solutions e.g., 2, 4, and 8 mL of a-Glu solution at 0.25 mg/mL concentration were replaced with equal volume of water used in synthesis process of p(NIPAM) cryogels keeping the other cryogel precursors the same. The scheme of the synthesis a-Glu@p(NIPAM) cryogels was demonstrated in **Figure 1 (a)** . During

the cryogel synthesis a-Glu enzymes were situated within pore walls or in pores of cryogels becoming engulfing or entrapped within p(NIPAM) cryogel network as cryopolymerization reaction occurred in the non-frozen regions about ice crystals at -20 °C. There are many advantages of using super cryogel network as smart bioreactors. Firstly, the desired amount of enzyme can be used to entrap within cryogels readily. Secondly, the possibility of activity loss during immobilization is minimized, as the polymerization and crosslinking reaction is carried out at -20°C where the enzymes are generally stored. Moreover, the conformational changes of enzyme that may lead to the activity loss as well as reaction of enzyme with the support somewhat is precluded in comparison to the other room temperature enzyme covalent linking reaction to a support material etc. The SEM images of thermo-responsive super porous p(NIPAM) cryogels, given in **Figure 1 (b)** clearly revealed that the cryogels have interconnected super porous structure with the pore size range of 1-100 μm. The super porosity of p(NIPAM) cryogels can provide a medium for higher diffusion and substrate exchange rates and rapid response to enzymatic reactions for a-Glu enzymes.

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Figure 1. (a) The scheme of a-Glu@p(NIPAM) cryogel system preparation, (b) SEM images of thermo-responsive super porous p(NIPAM) cryogels, and (c) the swelling% (S%) values of p(NIPAM) based cryogels at various temperatures.

The swelling% (S%) values versus solution temperature of p(NIPAM) and a-Glu@p(NIPAM) cryogel systems were investigated in 20-50°C intervals to determine the lower critical solution temperatures (LCST), and corresponding graph is presented in**Figure 1 (c)** . It was observed that the well-known LCST values of NIPAM that is 32 °C (Gandhi et al., 2015), shifted to 34.6±1.2 °C for p(NIPAM) based cryogels. The entrapments of a-Glu enzymes into p(NIPAM) cryogels has not significant effect the LCST values of p(NIPAM) cryogels. Therefore, it is rational to assume that the prepared p(NIPAM) cryogels possess hydrophilic properties at <34.6±1.2 °C possessing hydrogen bonding, and hydrophobic properties due to hydrophobic interaction at >34.6±1.2 °C, respectively. Moreover, the swelling, porosity, and pore volume% of p(NIPAM) and a-Glu@p(NIPAM) cryogel systems cryogels at 20 and 50°C were calculated and compared in **Table 1** . It is obvious that all p(NIPAM) based cryogels were shown higher S% at 20 °C than S% at 50 °C.

Table 1. The comparison of swelling. porosity, pore volume, and gel yield% values of super porous p(NIPAM), and a-Glu@p(NIPAM) cryogel systems.

Cryogel	Swelling %	Swelling %	Porosity %	Porosity %	Pore volume %	Pore volume %
	20 °C	**50 °C**	**20 °C**	**50 °C**	**20 °C**	**50 °C**
P(NIPAM)	1127±98	352±29	79.2±1.9	47.3±2.1	83.1±3.7	50.8±2.1
***@p(NIPAM)-0.5**	1116±112	353±34	78.7±1.4	45.6±3.1	80.6±2.5	48.2±3.2
***@p(NIPAM)-1**	1104±94	348±33	79.7±2.1	46.8±1.6	81.8±2.8	49.5±1.3
***@p(NIPAM)-2**	1111±126	324±49	77.3±2.7	46.1±1.7	81.3±1.5	50.1±1.8

*0.5, 1.0, 2.0 mg a-Glu enzyme/g of cryogels are denoted as @p(NIPAM)-0.5, @p(NIPAM)-1, and @p(NIPAM)-2, respectively.

It was apparent that there is no significant effect of entrapping of enzymes with different amounts into p(NIPAM) cryogels on the corresponding swelling% values for both 20 and 50 °C. The porosity and pore volume% of p(NIPAM) based cryogels were calculated and almost similar values e.g., around 80% at 20°C and 50% at 50 °C, respectively were found for all the cryogel systems. The higher swelling, porosity, and pore volume% values of p(NIPAM) based cryogels at 20°C can be explained by the fact that the temperature-responsive nature of p(NIPAM) cryogels as below LCSTs that is 34.6±1.2 °C, the p(NIPAM) cryogel chains

are hydrated due to hydrogen bonding that are much more than above LCSTs. The gel yields% of all the prepared cryogels was calculated as 90% indicates the efficient polymerization and crosslinking or NIPAM at cryogenic conditions.

The FT-IR spectra and TGA thermograms of p(NIPAM) cryogels and their corresponding a-Glu enzymes entrapped forms are given in **Figure 2** . As disclosed by the FT-IR spectra in **Figure 2 (a)** , the most distinct peaks for p(NIPAM) cryogel for C=O peaks at 1637 cm^{-1}, the N-H bending peaks of secondary amine at 1542 cm^{-1}, and the C-N stretching peak at 1473 cm^{-1} were present in a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogels systems. The increase in peak intensity at about 1044 cm^{-1} with the increase in the amount of a-Glu enzyme can be attributed to the more visible characteristic of the enzyme with higher concentration in the cryogel pores.

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Figure 2. (a) FT-IR spectra, (b) TGA thermograms of a-Glu@p(NIPAM) cryogel systems.

The thermal degradation of p(NIPAM) cryogel and the corresponding a-Glu enzyme entrapped forms were compared in **Figure 2 (b)** . The TGA thermograms were started directly from 25 °C due to possible denaturation of entrapped enzymes. The observed 7.1% weight loss up to 90 °C for bare p(NIPAM) cryogels can be attributed to intermolecular water or moisture removal. The increase on the weight loss as 8.6, 10.7, and 18.7% up to 90 °C for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogels systems can be also attributed to intermolecular water and lower thermal stability of enzymes (Demirci et al., 2020). Further increase of temperature increases the weight losses e.g., 90.4% weight loss was observed in 250-450 °C range, and 96% cumulative weight loss was measured at 750 °C for p(NIPAM) cryogels. On the other hand, a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems commenced to degrade in 100-170°C range with 23.3, 25.9, and 26.1% weight loses, respectively. The main degradation steps were observed in 210-440°C for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems as 83.8, 85.1, and 90.3% weight losses, respectively. Finally, 89.3, 91.5, and 94.3% of cumulative weight losses were observed for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems at 750 °C, respectively. So, overall, the thermal stability of a-Glu@p(NIPAM) cryogel systems are somewhat reduced comparable to lower temperatures in comparison to bare p(NIPAM) cryogel in about 75-350 °C range.

3.2 Enzyme leakage study

The advantages and disadvantages of various immobilization techniques were discussed and compared by many researchers (Boudrant et al., 2020; Reis et al., 2019; Sheldon and van Pelt, 2013). And, the most distinct advantage of entrapment method is no waste of enzymes arises as there is no binding is employed between enzyme and support that can decrease the activity of enzyme e.g., the use of active sites of enzymes to link the enzyme onto a matrix and some side reactions during immobilization and maybe some appropriate positioning of enzymes (Boudrant et al., 2020; Reis et al., 2019; Sheldon and van Pelt, 2013). On the other hand, the disadvantage of entrapment method is the ease of releasing or leakage of enzymes from support materials due to weakness of physical barrier and restrictions (Boudrant et al., 2020; Reis et al., 2019; Sheldon and van Pelt, 2013). The first step to observe possible enzyme leakage from the enzyme entrapped cryogels is reported by measuring the activity of washing solutions (Sheldon and van Pelt, 2013). Therefore, the washing solutions were tested in the enzyme assay to determine whether the enzyme leakage from p(NIPAM) cryogels is likely. Therefore, 20 µL of wash solutions of prepared a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were tested for the enzymatic reaction assay and corresponding graph is given in **Figure S1 (a)** . It was observed that, the absorbance value at 400 nm belong to 4-NP that is product of enzymatic reaction, was observed as 0.782±0.03 for free enzyme solution and 0.060±0.004, 0.083±0.004, and 0.127±0.006 for first washing solutions of a-Glu@p(NIPAM)-

0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems, respectively. Furthermore, the absorbance values at 400 nm for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were observed as 0.031±0.001, 0.051±0.002, and 0.100±0.003 for second washing solutions, and 0.011±0.005, 0.014±0.001, and 0.016±0.001 for third washing solutions, respectively. Also, the leakage% of enzyme form cryogel system were also calculated by comparing the measured absorbance values and after multiple washings and the results are given in **Table 2.** The absorbance values for free enzyme solutions were assumed as 100%.

Table 2. The leakage% of enzymes from relevant a-Glu@p(NIPAM) cryogel systems with multiple DD washing procedure.

Cryogel	**Leakage% of enzyme**	**Leakage% of enzyme**	**Leakage% of enzyme**
	I. washing	**II. washing**	**III. washing**
a-Glu@p(NIPAM)-0.5	6.8±0.6	3.1±0.2	0.6±0.1
a-Glu@p(NIPAM)-1	9.3±0.5	5.2±0.3	0.5±0.1
a-Glu@p(NIPAM)-2	15.1±0.8	11.7±0.3	0.9±0.1

After 1st washing of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1, and a-Glu@p(NIPAM)-2 cryogel systems the leakage% of enzymes as 6.8±0.6, 9.3±0.5, and 15.1±0.8% were calculated, respectively. The leakage% of enzymes from cryogel systems decreased to 3.1±0.2, 5.2±0.3, and 11.7±0.3% after 2nd washing step of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems, respectively and after 3rd washing of all a-Glu@p(NIPAM) cryogels <1% leakages were determined. Finally, the 4th washing solutions of -Glu entrapped cryogels were also tested in enzymatic assay and there is almost no absorbance peak at 400 nm was observed. Therefore, the onward studies were performed after 4-time washed a-Glu@p(NIPAM) cryogel system.

3.3 Enzymatic activity of a-Glu@p(NIPAM) cryogel systems

The calculations of enzymatic activity of free and a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were carried out using the reaction presented in **Figure S1 (b)** in accordance with the literature (Demirci et al., 2020). The a-Glu enzymes breaks the a-1,4 bonds in 4-NPG molecule to generate glucose and 4-nitrophenol (4-NP) molecules. Subsequently, the catalytic activity of the enzyme can be followed from the absorbance value of the generated 4-NP molecule that has a absorption maximum at 400 nm via UV-Vis spectroscopy. The reaction was carried out at pH 6.8 in PBS at 37 °C. Firstly, the enzymatic reaction was followed at different time intervals of reaction by using free a-Glu and a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel as biological catalyst. For the termination of free a-Glu used reaction, 2 mL of test solution were added into 2 mL 100 mM Na_2CO_3 solutions at 1st,3rd, 7th, 11th, 15th, 20th, 25th and 30th min of reactions. On the other hand, the a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were just removed from medium at 1st, 3rd, 7th, 11th, 15th, 20th, 25th and 30th min of reaction times, and the corresponding absorbance of 4-NP at 400 nm were followed. The corresponding absorbance of product vs time (min) graph is given in**Figure S1 (c)** . As can be seen that a linear increase occurred up to 20th min of reaction time when free a-Glu and/or a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems and become almost stable at 25th and 30th min of reaction. Therefore, the enzymatic reactions were terminated after 20 min.

In **Figure 3 (a)** , the activity values of free a-Glu enzymes in various amounts, and a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were compared at pH 6.8 in PBS at 37°C for 20 min rection time. The enzymatic activities of 0.01, 0.02, and 0.04 mg enzyme containing 40, 80, and 160 µL a-Glu solutions at 0.25 mg/mL concentration were calculated as 0.068±0.002, 0.049±0.0004, and 0.044±0.005 U/mL enzyme, respectively. It was also observed that the activities of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 are lower than activities of free a-Glu enzymes with the

calculated amounts of 0.045±0.002, 0.029±0.0006, and 0.018±0.0008 U/mL enzyme at pH 6.8 and 37 °C, respectively.

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Figure 3. The maximum activity of 3 different amount of enzyme load comparison of (a) activities and (b) activity % of a-Glu@p(NIPAM) cryogel systems with equivalent amounts of free enzyme. [Reaction condition: 5 mL of pH 6.8 buffer solution, 0.5 mL of 10 mM substrate, 37°C].

The activity% of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were calculated by assuming the calculated activity value for free a-Glu enzymes as 100% at pH 6.8 and 37 °C, and corresponding graph is given in**Figure 3 (b)** . The higher enzymatic activity was observed for 0.01 mg free enzyme (40 µL a-Glu solutions of 0.25 mg/mL concentration) with 0.068±0.002 U/mL enzyme was assumed as 100% activity. The activity% of 0.02, and 0.04 mg free enzymes (prepared by using 80, and 160 µL a-Glu solutions of 0.25 mg/mL concentration) were found to decreased to 72.8±0.6, and 65.4±6.7%. On the other hand, the activity% of a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were calculated as 66.2±3.3, 41.8±0.9, and 26.6±1.1%, respectively.

The most parameters to define the immobilized enzymes are immobilization yield, enzyme activity, and activity recovery (Boudrant et al., 2020; Reis et al., 2019; Sheldon and van Pelt, 2013). The immobilization yield, immobilization efficiency, and activity recovery% values were calculated for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems from washing solutions of enzyme@cryogel systems (un-entrapped enzymes) and the activity of used enzyme solutions for immobilizations at pH 6.8 by following literature (Boudrant et al., 2020; Sheldon and van Pelt, 2013), and summarized in **Table 3.**The term "immobilization yield" is usually used to describe the percentage of immobilized enzyme into support. The immobilization yield% for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were calculated as 89.4±3.1, 84.9±2.2, and 72.3±5.3%, respectively. It was observed that, the immobilization yield% decreased with the increasing amount of enzyme solutions used for immobilization into cryogels, and this can be explained with the entrapping capacity of p(NIPAM) cryogels as the more enzyme is used the more un-entrapment and/or non-efficient entrapment can occur. The terms of "immobilization yield" and "immobilization efficiency" are different from each other.

Table 3. The immobilization yield, immobilization efficiency, and activity recovery% values for a-Glu@p(NIPAM) cryogel systems with the respect to their free enzyme amounts.

Cryogel	Immobilization yield %	Immobilization efficiency %	Activity recovery %
a-Glu@p(NIPAM)-0.5	89.4±3.1	66.2±3.3	74.0±3.3
a-Glu@p(NIPAM)-1	84.9±2.2	57.4±0.9	67.5±0.9
a-Glu@p(NIPAM)-2	72.3±5.3	40.7±1.1	56.3±1.1

Different from "immobilization yield", the term "immobilization efficiency" is used to define percentage of immobilized enzyme activity according to equal amount of free enzyme (Sheldon and van Pelt, 2013). The 100% immobilization yield and 0% immobilization efficiency means that all enzymes are immobilized into support but there is no activity because of the deactivation or inaccessibility enzyme for some reason upon immobilization (Sheldon and van Pelt, 2013). The immobilization efficiency% values for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems were calculated as 66.2±3.3, 57.4±0.9, and 40.7±1.1% respectively assuming the activity% of the corresponding equal amounts of free enzymes as 100% at pH 6.8 and 37°C. The decrease in immobilization efficiency% with the increase in the entrapped enzymes within p(NIPAM) cryogels can be explain by the loss of enzymatic activity because of aggregation of enzymes

within carrier or by the in accessibility of substrates to enzymes within the carriers (Boudrant et al., 2020). The schematic presentation of possible enzyme aggregation with the increase in of amounts of entrapped enzymes in p(NIPAM) cryogels is demonstrated in**Figure 2S** . The enzymes are entrapped within the pores and pore walls of p(NIPAM) cryogels and the increase in the amounts of entrapped enzymes can cause to overload of enzymes and even shut down each other's active sides in the pore and pore walls. Moreover, the term "activity recovery" which is used for the definition of success of immobilization was calculated for a-Glu@p(NIPAM)-0.5, a-Glu@p(NIPAM)-1 and a-Glu@p(NIPAM)-2 cryogel systems and found as 74.0±3.3, 67.5±0.9, and 56.3±1.1% respectively, by assuming of activity% of corresponding equal amounts of free enzymes as 100% at pH 6.8 and 37°C.

As the higher activity% was observed for a-Glu@p(NIPAM)-0.5 cryogels system, this material, and its' equal amount of free a-Glu solution (40 µL of 0.25 mg/mL concentration) were used to determine the effect of temperature values of 15-50 °C range, and pH values of 4-9 range on activity%.

3.4 Effect of temperature and pH on enzymatic activity

Although many factors that affects the enzymatic activity positively or negatively have been reported, and amongst them temperature and pH stand out as the most prominent factors (Forsyth and Patwardhan, 2013; Hanefeld et al., 2009; Ho, 2012; Sheldon and van Pelt, 2013). Therefore, firstly, the effect of temperature on activity% of free a-Glu and a-Glu@p(NIPAM)-0.5 cryogels system were investigated at fixed pH value of 6.8 by changing the temperature of used reaction as 15, 20, 25, 30, 37, 45, and 50 °C. These temperature values were chosen to ensure the range covers the LCST of p(NIPAM) cryogels due its' thermo-responsive nature. The corresponding graph about the effect of temperature on activity% of free a-Glu and a-Glu@p(NIPAM)-0.5 cryogels system is given in **Figure 4 (a)** . The maximum activity% for free a-Glu enzyme was observed at 37 °C as 100% and was decreased with the increase or decrease in the temperature. The activity% of free a-Glu enzyme decreased to 70.4±7.8, 40.5±5.2, 18.6±1.1, and 5.2±0.5% at 30, 25, 20, and 15 °C, respectively. Similar decrease was also observed in the activity% of free a-Glu enzyme with the increase in the temperature to 45, and 50°C as 39.8±4.7 and 7.5±1.2% values were calculated, respectively. Interestingly, the activity% of a-Glu@p(NIPAM)-0.5 cryogels system were calculated more than 90% at 25 and 30°C with the activity% values of 98.4±0.2, and 92.8±2.4%, respectively. The activity% of a-Glu@p(NIPAM)-0.5 cryogels systems were decreased to 79.8±6.1, and 45.6±0.3% with the decrease of temperature to 20, and 15 °C, respectively. On the other hand, the activity% of a-Glu@p(NIPAM)-0.5 cryogels system were calculated as 66.2±3.3, 43.8±8.2, and 31.9±6.7% at 37, 45, and 50°C, respectively. The decrease on activity% of a-Glu@p(NIPAM)-0.5 cryogels above 30 °C can be explain with the increase hydrophobic nature of p(NIPAM) cryogels as this temperature value is above LCST values, 34.6±1.2 °C. As the p(NIPAM) cryogels shrunk above 34.6±1.2 °C, the entrapped enzyme microenvironment is somewhat protected. Overall, the p(NIPAM) cryogels above or below LCST protect the enzyme and lead the higher activity% for a-Glu@p(NIPAM)-0.5 cryogels in comparison to the free enzyme with exception of normal working temperature, 37°C.

The schematic presentation of possible cause of decreasing on activity% above 30 °C is demonstrated in **Figure 3S** . It is very well-known that the p(NIPAM) chains become dehydrated and hydrophobic coming close proximity with each other above LCST value causing overall shrinkage of p(NIPAM) cryogel network. Therefore, the decrease on activity% above 30 °C can be explain by overlapping and aggregation of the active sides of the entrapped enzymes. Also, the decrease in the swelling% value above LCST values due to increased hydrophobicity of p(NIPAM) cryogel can restrict the substrate access to deep inside cryogel network making the interaction of the substrate with enzyme molecules difficult causing a decrease in activity% of the entrapped enzyme.

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Figure 4. The effect of (a) reaction temperature, and reaction pH on the activity% of a-Glu@p(NIPAM)-0.5

cryogel systems and their comparison with the equal amounts of the free a-Glu enzymes at (b) 37, and (c) 25 °C reaction temperature. [Reaction condition (temperature): 5 mL of buffer solution at pH 6.8, 0.5 mL of 10 mM substrate; Reaction condition (pH): 5 mL of buffer solution, 0.5 mL of 10 mM substrate].

The activity% of free a-Glu is higher than a-Glu@p(NIPAM)-0.5 cryogel system at 37 °C. On the other hand, the entrapment of a-Glu enzymes within p(NIPAM) cryogels unveil improved thermal stability with higher activity% than free a-Glu enzymes at lower and higher temperatures. The obtained relatively better performance under rough reaction conditions e.g., lower or higher temperatures for a-Glu enzymes can be explained with the protection of -Glu enzymes within p(NIPAM) cryogel network attained by the immobilization process (Ding et al., 2016; Fan et al., 2016; Hamerska-Dudra et al., 2007; Pan et al., 2009; Wang et al., 2015).

The effect of pH on activity% of free a-Glu enzymes and a-Glu@p(NIPAM)-0.5 cryogel system were studied at 37 and 25°C, separately. For this purpose, the enzyme assays were carried out in PBS solutions at pH 4, 5, 6, 6.8, 8, and 9, respectively. The observed higher activity% was assumed as 100%. It was clearly seen from **Figure 4 (b)** , the higher activity% for free a-Glu enzymes were obtained at pH 6.8 and 37 °C and assumed as 100%. However, the activity% of a-Glu@p(NIPAM)-0.5 cryogel system was observed as 66.2±3.3% at pH 6.8 and 37°C. It was clearly seen from **Figure 4 (b),**the increase or decrease in medium pH has shown almost similar effect on activity of both free a-Glu and a-Glu@p(NIPAM)-0.5 cryogel system at 37°C. On the other hand, the higher activity% values were observed for a-Glu@p(NIPAM)-0.5 cryogel system at pH 6.8 at 25°C, whereas the activity% of free a-Glu enzymes observed as 40.5±2.8% at same conditions. Intriguingly, a-Glu@p(NIPAM)-0.5 cryogel system at any studied pH range (4-9) at 25°C reactions resulted in higher activity% than that free a-Glu enzymes. Interestingly, the activity% of free a-Glu enzymes was found decrease to less than 20% at pH 6, 5, and 4, and also decreased to less than 30% at pH 8 and above. On the other hand, a-Glu@p(NIPAM)-0.5 cryogel system maintained their 50% activity up to pH 5, and 65% activity up to pH 8 at 25 °C..

To sum up, the entrapping of a-Glu enzymes within p(NIPAM) cryogel can provide a-Glu enzyme an environment that a wide range of pH with higher enzyme activity% in comparison to the free a-Glu enzyme can be attainable. This statement is in agreement with the literature as the immobilization of enzymes into/onto a support can improved their stability at wide range of pH values (Gennari et al., 2020; Sirisha et al., 2016). In the immobilization process, the support material acts as a confined environment thanks to the cryogel network and the existing functional groups on the network. In this way, the temperature and pHs change can be substantially different inside and outside of network where enzymes can operate with higher activities (Rodrigues et al., 2013).

3.7 Kinetic parameters

Kinetic parameters of K_m (mM) and $V_{\max}$ (mM/min) were calculated from linear and non-linear plots of Michaelis-Menten equations using 5 mL PBS solution at pH 6.8, at 37 and 25 for free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel systems, respectively. The calculated $K_m$and $V_{\max}$ for free and entrapped enzymes by using 0.5 mL of 0.23-3.64 mM substrate solution were compared in **Table 4** . The K_m values were calculated as 1.34±0.03 and 1.07±0.02 mM from linear plots of Michaelis-Menten equation for free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system, respectively. On the other hand, the K_m values calculated from non-linear plots of Michaelis-Menten equation were slightly higher than the linear plots as 1.48±0.02, and 1.10±0.01 mM for free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system, respectively were computed.

Table 4. The comparison of kinetic parameters of a-Glu@p(NIPAM)-0.5 cryogel systems with the equal amount of free enzyme at 25 and 37 °C at pH 6.8.

Plots	**Parameters**	**25 °C**	**37 °C**
		Free a-Glu	**a-Glu@p(NIPAM)-0.5**
Linear	K_m **(mM)**	1.34±0.03	1.07±0.02

Plots	Parameters	25 °C	37 °C
Non-linear	V_{max} (mM/min)	0.004±0.0003	0.002±0.0002
	K_m (mM)	1.48±0.02	1.10±0.01
	V_{max} (mM/min)	0.010±0.005	0.002±0.0001

Moreover, the V_{max} values for free a-Glu enzyme were calculated as 0.004±0.0003, and 0.010±0.005 mM/min from linear and non-linear plots of Michaelis-Menten equations, respectively. And the V_{max} values of a-Glu@p(NIPAM)-0.5 cryogel system were also calculated and the same value of 0.002±0.0001 mM/min for linear and non-linear plots of Michaelis-Menten equations, respectively was obtained.

The curve fitting graphs of the linear and nonlinear Michaelis-Menten equations of both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system were given in **Figure S4 (a)** , and **(b)** , respectively. It was clearly seen from **Figure S4** that the obtained K_m and V_{max} from nonlinear Michaelis-Menten equation was shown better fit with the experimental results. This can be explained with the reported disadvantage of linearized Michaelis-Menten equations that results in poor estimation of K_m and V_{max} as it compresses data points at high substrate concentrations into a small region and highlights data at lower substrate concentrations (Cho and Lim, 2018; Hernández and Ruiz, 1998; Kemmer and Keller, 2010; Marasović et al., 2017).

In overall, the calculated K_m and V_{max} values for free a-Glu enzyme as 1.48±0.02 mM and 0.010±0.005 mM/min are higher than a-Glu@p(NIPAM)-0.5 cryogel system. As the affinity of free enzyme to substrate maybe lower than entrapped ones due to functional groups of the cryogels that may adsorb the substrate readily and then directed to the active sites of the a-Glu in situ instantly in comparison to the free -Glu enzyme (Sahoo et al., 2013; Sun et al., 2012). It is also confirmed that the higher V_{max} values of free a-Glu enzymes than a-Glu@p(NIPAM)-0.5 cryogel system at relevant temperatures affirms that faster reaction rates for free enzyme yet lower activity% values.

3.6 Stability in reuse and storage study

The main purpose of development of immobilization techniques of enzymes into/onto support materials is to achieve improvement of stability of enzymes at harsh conditions, and also the creation of reusable forms of enzymes to reduce the cost of enzymatic reactions (Jesionowski et al., 2014; Jia et al., 2014; Sheldon et al., 2005). Therefore, the reusability of both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system were investigated at pH 6.8 buffer solution and 25, and 37°C. The activity% of first use assumed as 100%, and both free and entrapped enzyme systems were washed with buffer solution at pH 6.8 for 30 min. The decreasing on activity% in reusability studies of free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system at 25°C is given in **Figure 5 (a)** . The activity% of free a-Glu enzyme were calculated 36.9±7.1% at 2^{nd}use and decreased less than 15% after 3^{rd} use. On the other hand, the activity% of a-Glu@p(NIPAM)-0.5 cryogel system at 25°C were calculated as 96.3±0.4, and 90.2±0.8% at 2^{nd}, and 3^{rd} uses, respectively. Also, a-Glu@p(NIPAM)-0.5 cryogel system maintained almost 50% activity after 10 successive use at 25 °C.

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Figure 5. The comparisons of the reusability of free a-Glu enzyme and a-Glu@p(NIPAM) cryogel systems at (a) 25°C, and (b) 37 °C. [Reaction condition: 5 mL of buffer solution at pH 6.8, 0.5 mL of 10 mM substrate].

The reusability of free and entrapped enzyme systems at 37°C were also demonstrated in **Figure 5 (b)** . The activity% of free a-Glu enzymes were found decrease to less than 10% after 4^{th} use. On the other hand, the activity% of a-Glu@p(NIPAM)-0.5 cryogel system maintained 70% activity after 7 sequential use, and almost 50% activity after 10 consecutive use. It is obvious that the entrapment of a-Glu enzymes within

p(NIPAM) cryogels provide reusability of enzymes with high activity% at both 25 and 37°C in comparison to their free forms at the same temperatures.

The change on activity% values of free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system after storing at various temperature were tested for 10 days, and corresponding graphs are given in**Figure 6** . Both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system were stored at -20, +4, and 25 °C for 10 days and at the end of these times their storage stabilities were compared. The enzymatic reaction was carried out at 37 and 25 °C for both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system, respectively, and the activity% of first usages were assumed as 100%.

In **Figure 6 (a)** and **(b)** , the calculated activity% values of free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel system stored at -20 °C are given for 37, and 25°C reaction temperature, respectively. It was clearly seen from **Figure 6 (a)** that both free and a-Glu@p(NIPAM)-0.5 cryogel system maintained their about 50% activity at 37°C reaction temperature after 10-day storage at -20°C. On the other hand, as seen in **Figure 6 (b)** , almost 60% activity was maintained by a-Glu@p(NIPAM)-0.5 cryogel system and there is almost no activity observed for free a-Glu enzymes at 25 °C reaction temperature after 10-day storage at -20 °C.

The activity% for both free a-Glu enzyme and a-Glu@p(NIPAM)-0.5 cryogel systems were also investigated at 37 and 25 °C reaction temperature during the 10-day storage at +4°C, and corresponding graphs are given in**Figure 6 (c), and (d)** , respectively. In Figure 6 (c), the activity% of free a-Glu enzyme was decreased to around 80% after 5-day storage, whereas the activity% of a-Glu@p(NIPAM)-0.5 cryogel systems was decreased to less than 60% at 37 °C reaction temperature. After 10^{th} day at +4°C, the activity% for free a-Glu and a-Glu@p(NIPAM)-0.5 cryogel systems were observed as 52.4±1.6, and 32.9±1.2% at 37 °C reaction temperature, respectively.

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Figure 6. The comparisons of the storage stability of free a-Glu enzyme and a-Glu@p(NIPAM) cryogel systems (a) stored at -20°C with the reaction temperature of 37°C, (b) stored at -20 °C with the reaction temperature of 25 °C, (c) stored at +4°C with the reaction temperature of 37°C, (d) stored at +4 °C with the reaction temperature of 25 °C, (e) stored at +25°C with the reaction temperature of 37°C, and (f) stored at +25 °C with the reaction temperature of 25 °C. [Reaction condition: 5 mL of buffer solution, 0.5 mL of 10 mM substrate].

Moreover, after the storage of free a-Glu and a-Glu@p(NIPAM)-0.5 cryogel system at 25 °C for 10 days, the activity% values were calculated, and corresponding graphs are given in **Figure 6 (e), and (f)** for 37 and 25 °C reaction temperature, respectively. It was observed from 37 °C reaction temperature in **Figure 6 (e)** that, the activity% of both free a-Glu and a-Glu@p(NIPAM)-0.5 cryogel system was decreased to less than 10% after 10-day storage at 25 °C. In **Figure 6 (f)** , similarly with 37 °C reaction temperature there is almost no activity was observed for free a-Glu enzyme at 25°C reaction temperature after 10-day storage at 25°C. On the other hand, after 10-day storage at 25°C, almost 50% activity was preserved by a-Glu@p(NIPAM)-0.5 cryogel system at 25 °C reaction temperature. It is apparent that the use of cryogels matrices as enzyme compartment vehicles increases the storage stability of a-Glu enzyme, and also the conditions of free -Glu enzyme can be easily shifted from sub-zero temperature to room temperature by using super porous smart p(NIPAM) cryogels.

3.8 Inhibition studies

Among the significance pathways to control type II diabetes and hyperglycemia, the inhibition of a-Glu enzymes has attracted great attention in literature (Nair et al., 2013; Yang et al., 2019). The inhibition of a-Glu enzymes can lead to decrease in blood sugar level as this is a main problem for hyperglycemia and type

II diabetes (Kwon et al., 2006). Therefore, the change in the possible inhibition profiles of free a-Glu enzymes and a-Glu@p(NIPAM)-0.5 cryogel system was investigated at pH 6.8 for the reaction temperatures of 37 and 25°C, and corresponding graph is given in **Figure 7** . The RA solutions at various concentrations were added to reaction medium to see inhibition power on enzymatic activity of both free and a-Glu@p(NIPAM)-0.5 cryogel system.

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Figure 7. The inhibition% profile of free a-Glu enzyme and a-Glu@p(NIPAM) cryogel systems at (a) 37, and (b) 25°C reaction temperatures. [Reaction condition: 4 mL of buffer solution, pH:6.8, 0.5 mL of 10 mM substrate, 1 mL of inhibitor solution in relevant buffer solution].

The inhibition profile of free and entrapped -Glu enzyme at 37°C reaction temperature is shown in Figure 7 (a). It was clearly seen that the inhibition% values were increased with the increase in the concentration of RA solution. The 100% inhibition was observed at 1.8 mg/mL concentration of RA solution for both free and entrapped -Glu enzyme at 37 °C. However, the higher inhibition% values was observed for a-Glu@p(NIPAM)-0.5 cryogel system at lower concentration of RA solution, and this can be explained with the higher activity% of enzyme at 37 °C. The graph of inhibition studies at 25 °C is presented in Figure 7 (b). It was clearly seen that the free a-Glu enzymes can be inhibited at lower RA concentration then than a-Glu@p(NIPAM)-0.5 cryogel system. However, almost 100% inhibition was observed at 1.8 mg/mL concentration of RA solution for both free a-Glu and a-Glu@p(NIPAM)-0.5 cryogel system. At 1.2 mg/mL concentration 81.6% inhibition of a-Glu@p(NIPAM)-0.5 cryogel system was attained whereas 100% inhibition of free a-Glu enzyme was observed. Again, confirming a shelter and/protection housing ability of p(NIPAM) cryogels for a-Glu enzymes.

4. Conclusion

The use of super porous thermo-responsive p(NIPAM) cryogels as a support for enzyme immobilization shown to be very simple, effective and versatile e.g., the amounts of entrapped enzymes within cryogels can easily increase with the increase in the amount enzyme added into the cryogel precursor solution during cryogel synthesis. Interestingly, it was found that the increase in the amounts of entrapped enzymes within p(NIPAM) cryogels decreased the activity% attributed to the certain capacity of cryogel to reside the enzymes without overlapping or aggregation within cryogels network. The entrapment of enzymes in p(NIPAM) cryogels did not affect the LCST of cryogels as 34.6±1.2°C value measured for bare and enzyme entrapped p(NIPAM) cryogels. The optimum working temperature for a-Glu@p(NIPAM)-0.5 cryogel system was determined as 25°C which is lower than free a-Glu enzyme's that is 37°C. This is a significant step forward for industrial utilization of enzymes in terms of offering lesser energy consumptions. The decrease on optimum working temperature of a-Glu enzyme after entrapping into p(NIPAM) cryogels attributed to chemical structure of the corresponding thermo-responsive p(NIPAM) cryogels that possess changeable hydrophilic and hydrophobic behavior e.g., below LCST and p(NIPAM) cryogel is hydrophilic whereas it is hydrophobic above LCST values. Moreover, the stability of entrapped a-Glu enzymes at wide range of pH e.g., at pH 6-8 range with more than about 70% activities can be reached that is significantly higher than free a-Glu enzymes at relevant pH values. The entrapment of a-Glu enzymes within p(NIPAM) cryogels also makes the possible reusability as more than 50% activity keeping at the end of 10 consecutive practices at 25 and 37 °C was measured. Additionally, the storage stability of a-Glu enzymes was increased with the entrapment within p(NIPAM) cryogels as more than 50% activity values in 10 days storage at -20, +4, and even 25°C were observed. These kinds of thermo-responsive p(NIPAM) cryogel matrices can be successfully employed for also different enzymatic reactions, e.g., the hydrolysis of reactions of -1,4-linked glucose residues from aryl (or alkyl) -glycosides, disaccharides or oligosaccharides. Therefore, the use of smart stimuli cryogel networks with tunable porosity, and pore sizes in response to temperature can be suitable tool for the

entrapment of other enzymes in novel biotechnological uses and industrial applications.

Acknowledgement

The authors gratefully acknowledge financial supports provided by the Scientific Research Commission of Canakkale Onsekiz Mart University (FDK-2020-3437), and a scholarship for Sahin Demirci from Council of Higher Education System (YOK-100/2000).

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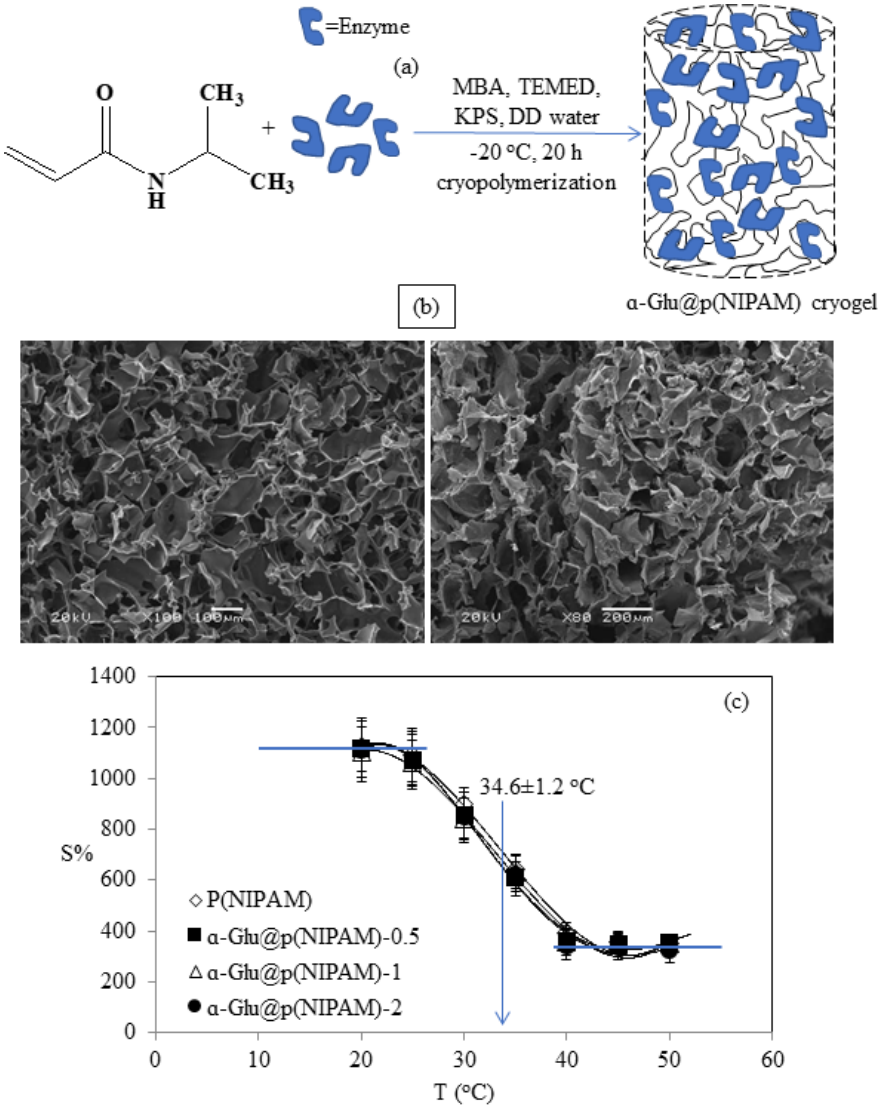

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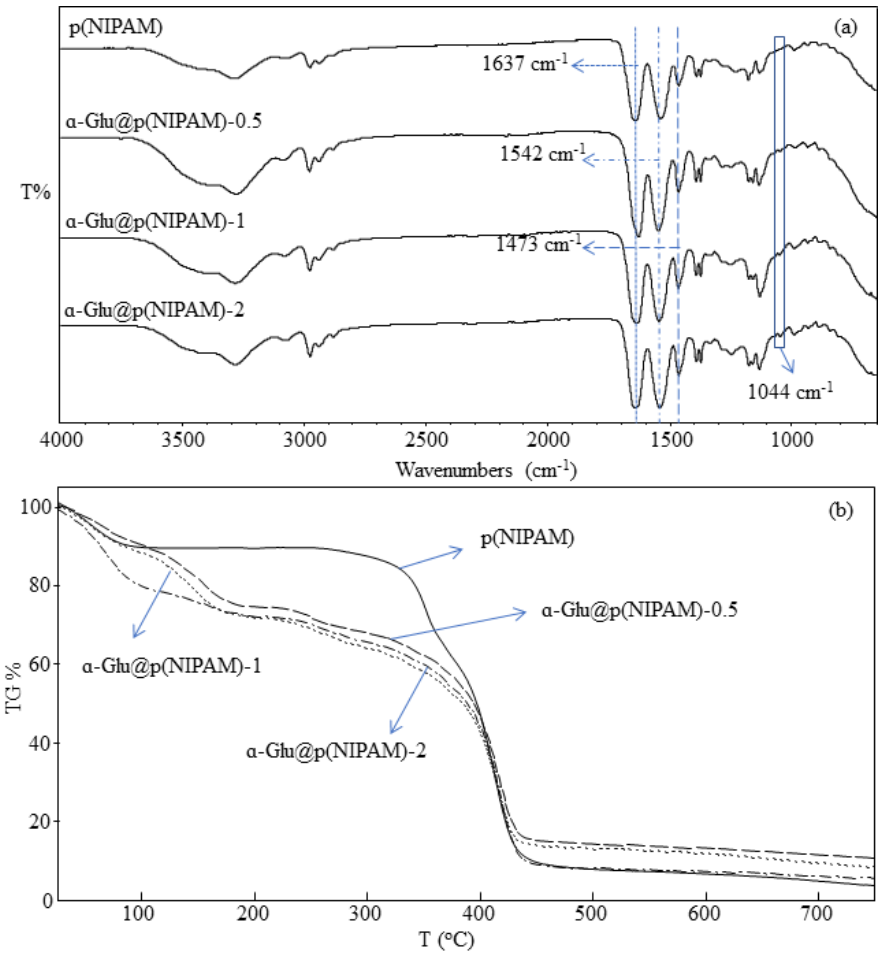

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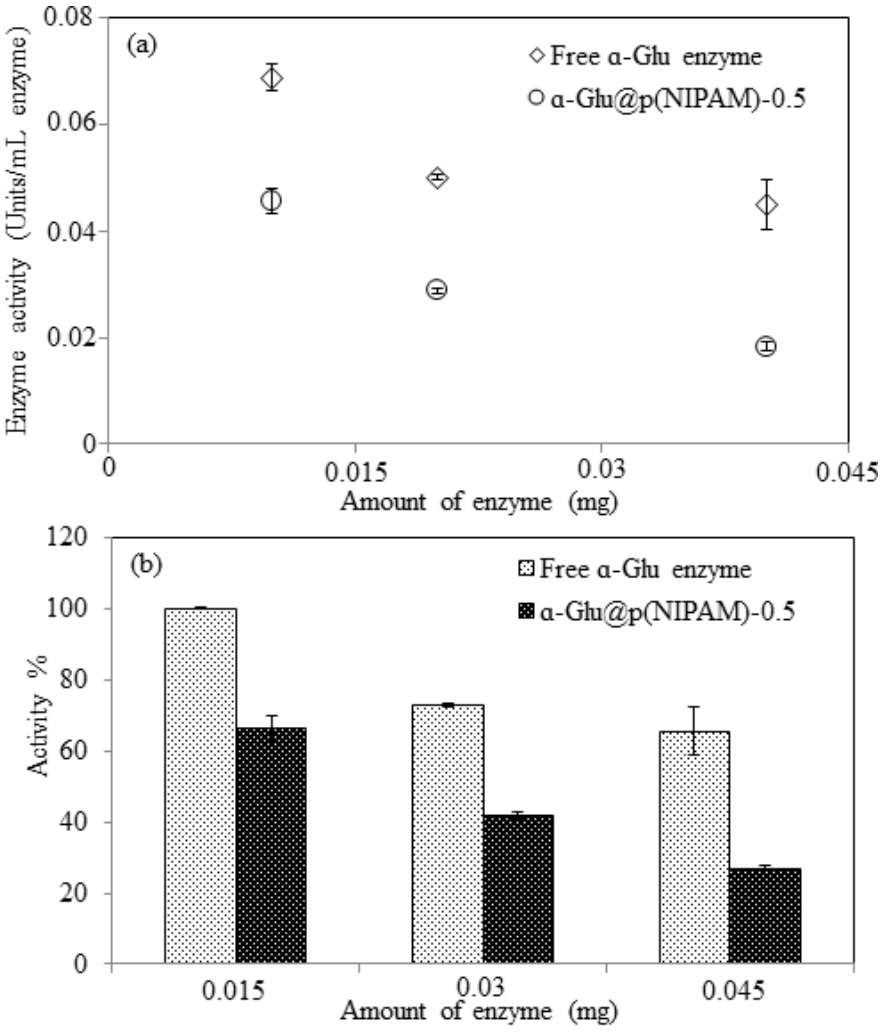

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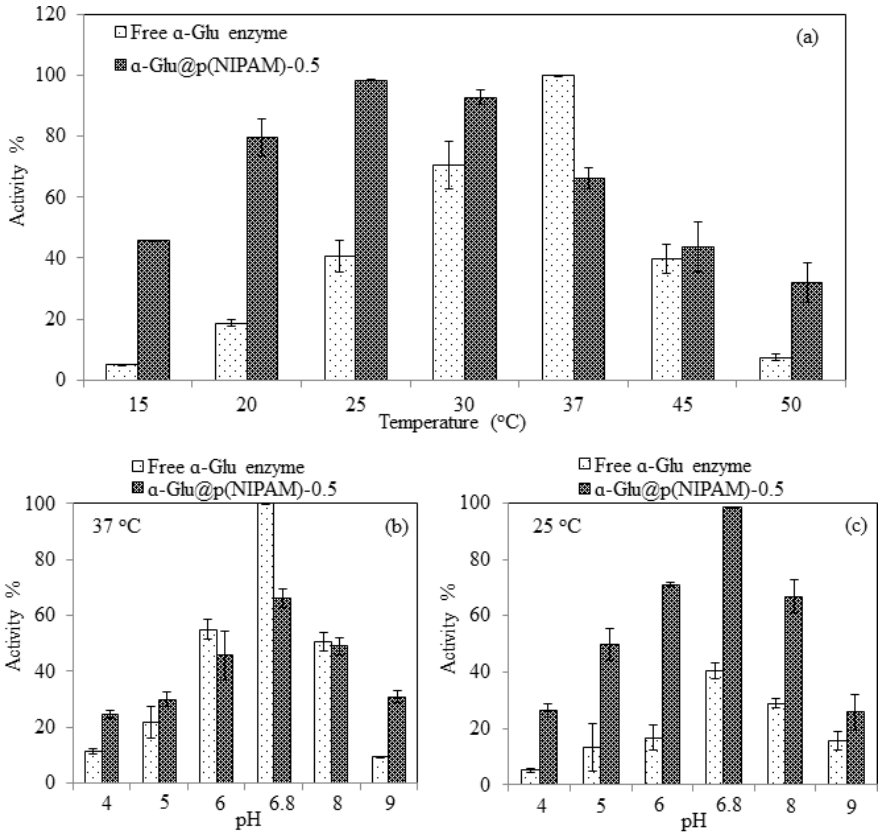

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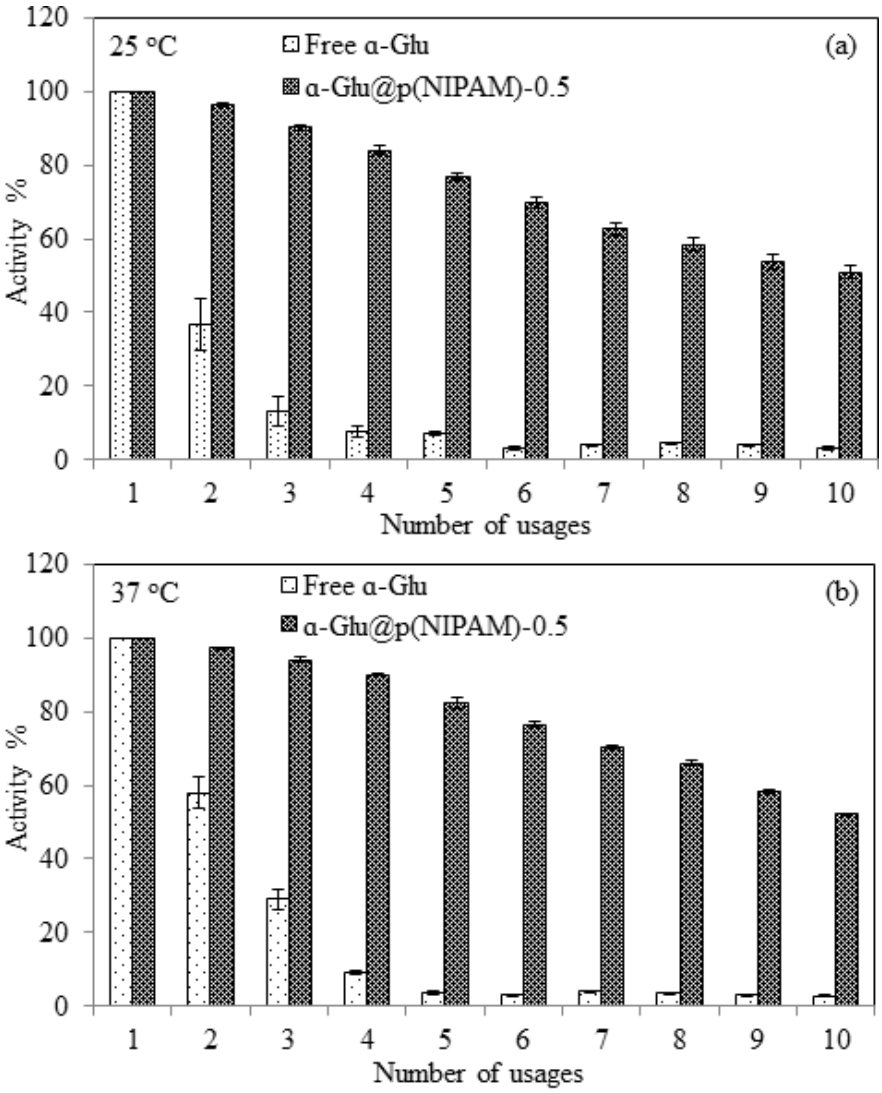

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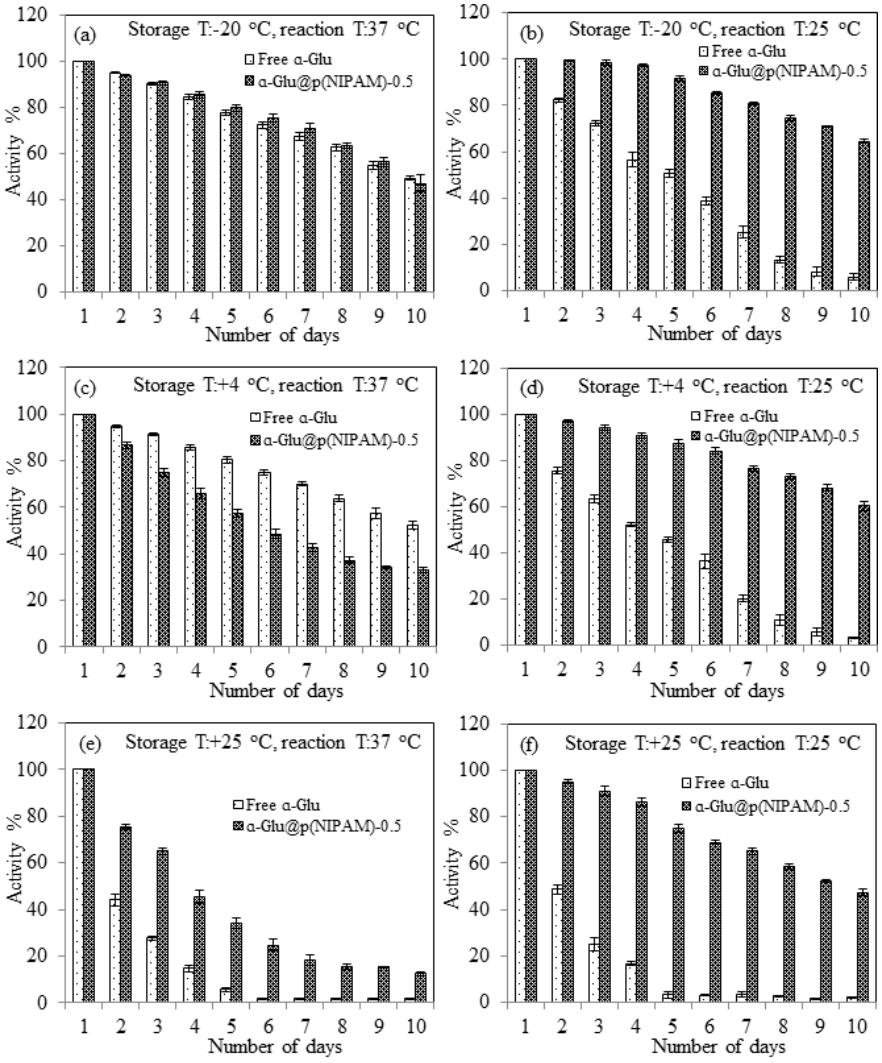

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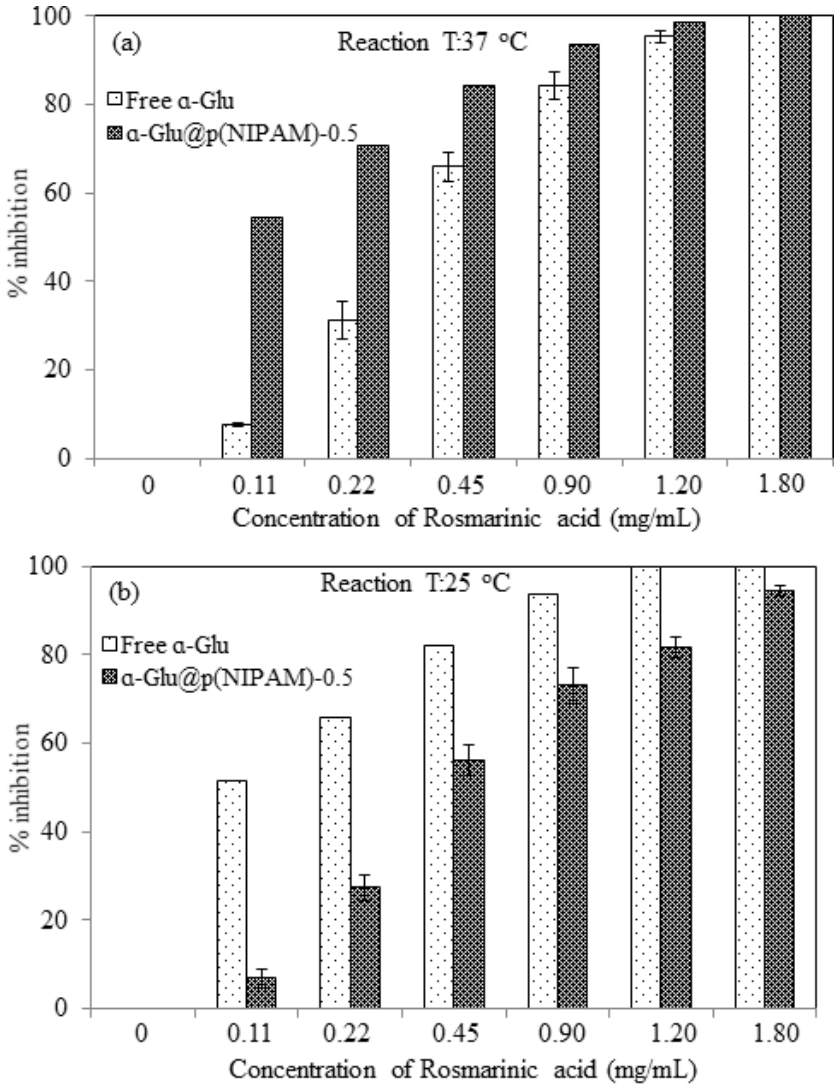

Figure 7.

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