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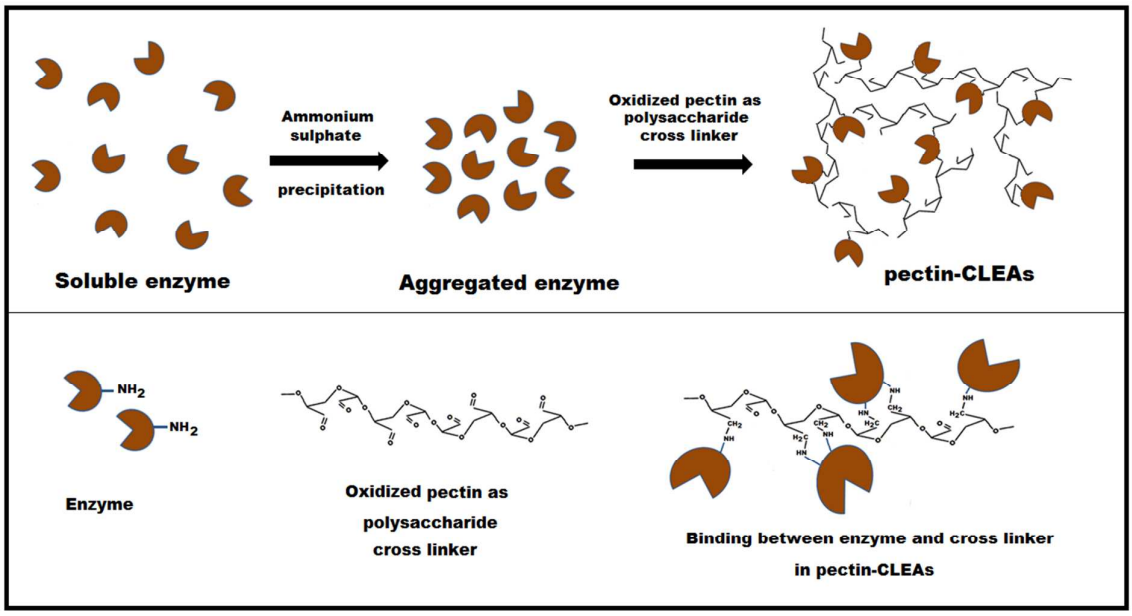
CLEAs of glucoamylase were prepared using pectin as non-toxic and macromolecular cross-linker which showed improved thermal stability and good reusability.



Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase†

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†Electronic supplementary information (ESI) available: Original and second derivative FTIR spectra of free enzyme and pectin-CLEAs

Abstract

Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase were prepared for the first time with pectin as cross-linking agent. Pectin as biocompatible, biodegradable, non-toxic, renewable and macromolecular cross-linker was used instead of traditional micro-molecular glutaraldehyde cross-linker. Cross-linker was prepared by controlled sodium metaperiodate oxidation of native pectin. The effects of precipitant type, amount of precipitant and cross-linking on activity recovery of glucoamylase in pectin-CLEAs were studied. After aggregation of glucoamylase with ammonium sulphate, when formed aggregates were cross-linked by pectin, 83% activity recovery was achieved in pectin-CLEAs, whereas when cross-linked by traditional cross-linker glutaraldehyde, 64% activity recovery was achieved in glutaraldehyde-CLEAs. After formation of pectin-CLEAs and glutaraldehyde-CLEAs, optimum temperature for glucoamylase activity was shifted from 50 to 55°C. The free enzyme and pectin-CLEAs displayed optimal pH 5, whereas optimal pH of glutaraldehyde-CLEAs was shifted to pH 6. Compared with free enzyme and glutaraldehyde-CLEAs, lower inactivation rate constant of glucoamylase in pectin-CLEAs within the temperature range of 50-70°C was observed. Moreover, activation energy required for denaturation of glucoamylase in pectin-CLEAs was higher than glutarldehyde-CLEAs and free enzyme. Kinetic studies show that the K_m and V_{max} of glucoamylase remained unchanged after pectin-CLEAs formation, whereas K_m was increased and V_{max} was decreased after glutaraldehyde-CLEAs formation. Finally upon 10 consecutive uses, pectin-CLEAs retained 55% initial activity and glutaraldehyde-CLEAs retained only 29% initial activity. These results suggest that this pectin-CLEA is potentially usable in industrial applications.

Keywords: Enzyme immobilization; Cross-linked enzyme aggregates (CLEAs); Glucoamylase; Pectin

Introduction

In the food and beverage industries, glucoamylase (EC 3.2.1.3) is used for saccharification of oligosaccharides and starch.[1] However; enzymes are usually expensive and often not sufficiently stable under industrial process conditions. To address this, enzyme immobilization has been developed as tool for stabilization, separation and reutilization of enzymes, thus decreases the price of this relatively expensive compound.[2-4]

In recent years, carrier free immobilization of enzymes as cross-linked enzyme aggregates (CLEAs) has raised increasing interests due to its simplicity in preparation and robustness of the immobilized enzymes.[5] CLEAs present several interesting features such as highly concentrated volumetric activity and space time yield, high stability against denaturing agents, high enzyme activity in aqueous and organic media, low production cost due to exclusion of carrier, amenability to easy scale-up, easiness of synthesis, excellent recoverability/repeated usage, and the fact that no purified enzymes are needed.[6-7] To immobilize enzyme as CLEAs, the enzyme is aggregated by the addition of organic solvents[8-9], non-ionic polymers[10-11] and inorganic salts [12-13] to an aqueous solution of enzyme. In a subsequent step, the physical aggregates of the enzymes are cross-linked with glutaraldehyde as cross-linker. During cross-linking, enzyme molecules are bound together via the covalent bond between the bi-functional aldehyde groups on glutaraldehyde and the numerous amino groups on enzyme molecules. Thus cross-linking step gives a more stable superstructure to the aggregates by establishing covalent bonds between enzyme molecules, which render them permanently insoluble.[14]

Traditionally, among the many available protein cross-linking agents, glutaraldehyde has been undoubtedly used as the cross-linking agent to prepare CLEAs. It is a powerful cross-linker which remained as one of the most interesting tools in enzyme cross-linking and

immobilization.[15] Thus, although the glutaraldehyde is considered of general applicability for cross-linking in CLEAs, the cross-linking step may be complicated in case of some enzymes. For instance, in case of nitrilases CLEAs, low or no retention of activity has been observed when glutaraldehyde was used as the cross-linker. It is due to the small size of the glutaraldehyde which allows it to penetrate the interior of the enzyme, causing the reaction of the cross-linker with amino groups that are crucial for the catalytic activity of the enzyme.[16] Due to extremely smaller molecular length of glutaraldehyde than enzymes, it can get access to all amino groups of enzymes and form more compact structure of CLEAs. This brings about special accessibility problem for macromolecular substrates into CLEAs resulting in serious mass transfer limitations especially when enzymes are acting on macromolecular substrates.[17] In addition, glutaraldehyde presents many toxic effects on aquatic species.[18] Therefore, the utilization of glutaraldehyde for the formation of CLEAs destined for environmental applications can pose a problem because it can possibly leach from the CLEAs to the receiving environment where it can cause toxic effects to the aquatic ecosystems.

In order to overcome these problems, recently some researchers have attempted to replace glutaraldehyde as a cross-linking agent. Arsenault *et al.*[19] used biocompatible and biodegradable biopolymer chitosan as a novel cross-linking agent for the preparation of laccase CLEAs to avoid the adverse effects on aquatic ecosystems caused by leaching of glutaraldehyde during the treatment of water contaminated by the endocrine disrupting chemicals. Glucose oxidase, peroxidase and urease were cross-linked with a biocompatible and non-toxic biomolecule L-Lysine as the cross-linker in the achievement of alternative and biocompatible enzyme aggregates.[20] Hydroxyl groups of serine and threonine residues in proteins were oxidized with periodate to yield aldehydes and then cross- linking was carried out by adding amino acid L-Lysine. To enhance the mass transfer in CLEAs, porous β-

mannanase CLEAs were prepared using linear dextran polyaldehyde as a macromolecular cross-linker instead of micro-molecular glutaraldehyde.[21] Due to more length of dextran polyaldehyde than glutaraldehyde, structure of β-mannanase CLEAs was enlarged leading to pore formation. These β-mannanase CLEAs exhibited sixteen times higher activity on macromolecular substrates than prepared by glutaraldehyde. Moreover, due to the large size of dextran polyaldehyde, it can't penetrate the interior of the enzyme which precludes its possible reaction with catalytically relevant amino groups in the active cleft. More recently, Velasco-Lozano *et al.*[22] developed an interesting approach of carboxyl-CLEAs via cross-linking of carboxyl groups with polyethyleneimines as cross-linker to avoid inactivation of enzyme due to glutaraldehyde cross-linking with catalytically relevant amino groups in the active cleft. The carboxyl-CLEA preparation was carried out by first activating the carboxyl groups of the enzyme by the addition of 1-ethyl-3-[3-dimethylaminopropil] carbodiimide and N-hydroxysuccinimide followed by cross-linking of activated carboxyl groups through primary amino groups of the polyethyleneimine.

In line with these attempts to replace glutaraldehyde, here we report for the first time polysaccharide based macromolecular cross-linker for the preparation of CLEAs. Polysaccharide based cross-linkers have received an increasing attention as an ideal cross linking agent of proteins in recent years.[23] They are produced by controlled sodium metaperiodate oxidative cleavage of the C-2 and C-3 bond of the monomeric units of native polysaccharide resulting in the formation of aldehyde groups. The aldehyde groups can then cross-link with ε-amino groups of lysine or hydroxyl lysine side groups of protein by Schiff's base formation.[24] Pectin is a high-molecular weight, biocompatible, non-toxic and natural polysaccharide. In the present study, oxidized pectin with aldehyde groups was successfully prepared by periodate oxidization. Then we have explored the feasibility of the oxidized pectin as polysaccharide cross-linker for the preparation of pectin-cross linked enzyme

aggregates (pectin-CLEAs) of glucoamylase. The effects of precipitation and cross-linking on the enzyme activity recovery were investigated. The optimal catalytic temperature and pH, thermal stability of pectin-CLEAs and kinetic parameters were measured. Lastly, the reusability of the pectin-CLEAs was also measured.

Experimental

Materials

Glucoamylase (OPTIDEX L-300) was gifted by Riddhi Siddhi Gloco Biols Ltd. (Gokak, India). Ammonium sulfate, acetone, n-propanol, ethanol, acetonitrile were purchased from S.D. Fine Chemicals, Mumbai. Pectin (M_w = 75 kDa, degree of esterification = 65-70%, galacturonic acid = min. 65%) was purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Sodium metaperiodate was obtained from S.D. Fine Chemicals, Mumbai. Glucose oxidase-peroxidase (GOD-POD) kit was purchased from Accurex Biomedical Pvt. Ltd. Mumbai, India. All other chemicals were of analytical grade and procured from reliable sources.

Determination of enzyme activity

Glucoamylase activity was estimated using maltodextrin as a substrate.[25] For both free and immobilized enzyme, enzyme samples were added to 1% maltodextrin solution prepared in 0.1 M acetate buffer of pH 5. Mixture was kept at 50 °C for incubation; after 15 min it was kept on ice and 5% tricarboxylic acid was added to stop the reaction. The released glucose was detected by a glucose oxidase-peroxidase (GOD-POD) kit.[26] Above sample (20 µL) was added to GOD-POD working enzyme solution (2 mL) and kept for 30 min at room temperature (~28±2°C). The absorbance was measured at 505 nm using UV-Vis spectrophotometer (Hitachi U2001, Japan). The glucoamylase activity was calculated using a

standard curve plotted using glucose in the range of 0.0-1.0 mg/mL. One unit (U) of enzyme activity is defined as the amount of enzyme required to release 1µmol of glucose per minute at 50°C and pH 5.

Preparation of pectin cross-linker

Oxidized pectin as polysaccharide cross-linker was prepared according to Gupta *et al.*[27]. Pectin (2 g) was dissolved in distilled water containing 20% (v/v) ethanol. To this solution, 3 mL of 0.5 M sodium metaperiodate was added and the pH of the solution was maintained at 3.5 using dilute hydrochloric acid and sodium bicarbonate solution. The resulting solution was kept in dark under constant stirring for 2 h at 30 °C. After 2 h, the oxidized pectin was precipitated out in excess isopropanol. The oxidized product was separated using vacuum filtration. Generation of aldehyde groups on oxidized pectin was confirmed by Fourier transform infrared (FTIR) spectroscopy using Shimadzu IRAffinity-1 FTIR Spectrophotometer. Oxidized pectin solution was prepared (40 mg/mL) in 0.1 M acetate buffer of pH 5 and used as cross-linker for the preparation of pectin-CLEAs.

Preparation of glucoamylase pectin-CLEAs

Precipitation of enzyme

Chilled organic solvents (methanol, ethanol, isopropanol, n-Butanol, acetone, acetonitrile 10 mL each) and saturated ammonium sulphate solution (10 mL) were added drop wise separately to samples of free glucoamylase (2 mL containing 530 U of glucoamylase and protein content 4 mg/mL) with shaking and kept for 30 min at 4°C for complete precipitation of enzyme and then centrifuged for 10 min at 10,000×g. The precipitates and supernatants were collected separately. The precipitate was redissolved in 0.1M sodium acetate buffer, pH

5. The glucoamylase activity was then checked in both supernatants and aliquots of redissolved precipitate.

Cross-linking of enzyme with pectin

Saturated ammonium sulphate solution (10 mL) was added to glucoamylase (2 mL containing 530 U of glucoamylase and protein content 4 mg/mL) in capped centrifuge tube. After keeping the mixture for 30 min at 4°C for complete precipitation of enzyme, oxidized pectin was added to a final concentration of 1.5 % (v/v). The mixture was kept at 30°C for 16 h with constant shaking at 150 rpm. Then the suspension was centrifuged at 10,000×g for 10 min. The supernatant was collected and checked for glucoamylase activity. The pellet was washed three times with 0.1 M acetate buffer of pH 5 to remove unreacted oxidized pectin and unbound proteins. The final enzyme preparation was kept in the same buffer (1 mL) at 4°C. The percent activity recovery of glucoamylase in pectin-CLEAs was determined using following equation:

$$Activity\ recovery\ (\%) = \frac{Total\ activity\ of\ glucoamylase\ in\ CLEAs\ (U)}{Total\ activity\ of\ glucoamylase\ used\ for\ CLEAs\ preparation\ (U)} \times 100$$

Preparation of glutaraldehyde-CLEAs (GA-CLEAs) of glucoamaylase

Traditionally, glutaraldehyde is the cross-linker choice during preparation of CLEAs. Hence, to compare usefulness of new pectin cross-linker, GA-CLEAs were also prepared. The above mentioned procedure of pectin-CLEAs preparation was repeated to prepare GA-CLEAs of glucoamylase, where for the cross-linking glutaraldehyde was used as cross-linker. Cross-linking was done by using glutaraldehyde to a final concentration of 1.5 % (v/v) and keeping

cross-linking time 16 h. The percent activity recovery of glucoamylase in GA-CLEAs was determined by using same equation which was used for pectin-CLEAs.

Molecular secondary structure studies of pectin-CLEAs by FTIR method

Changes in secondary structure of glucoamylase after pectin-CLEA preparation was analysed by Fourier transform infrared (FTIR) analysis. For FTIR analysis, the infrared spectra of free enzyme and pectin-CLEA were recorded by Shimadzu IRAffinity-1 FTIR Spectrophotometer from 4000 to 400 cm^{-1} with samples powder dispersed in the pressed KBr discs. Peak frequencies in amide I region (1600-1700 cm^{-1}) were identified using the secondary derivative. Then according to Wang *et al.*[28] a multi-peak fitting program with Gaussian function in Origin 8.0 was used to quantify the multicomponent peak areas in amide I bands of free enzyme and pectin-CLEAs. The relative fractions of β-sheets (1613-1640 cm^{-1}, 1682-1689 cm^{-1}), random coil (1640-1645 cm^{-1}), α-helix (1645-1662 cm^{-1}) and β-turn(1662-1682 cm^{-1}) based on the modelled peak area were calculated according to the report generated by the software.[29-30]

Optimal conditions for enzyme activity

The effect of temperature on glucoamylase activity of free enzyme, GA-CLEAs and pectin-CLEAs was determined in the temperature range of 30-80°C at pH 5. The effect of pH on glucoamylase activity of free enzyme, GA-CLEAs and pectin-CLEAs was studied at 50°C in the pH range of 3-9 using 0.1 M buffers (pH 4-5, sodium acetate buffer; pH 6-8, sodium phosphate buffer; pH 9, NaOH/glycine buffer). Finally optimum temperature and pH required for maximum glucoamylase activity of free enzyme, GA-CLEAs and pectin-CLEAs were determined.

Thermal stability study

Thermal stabilities of free enzyme, GA-CLEAs and pectin-CLEAs were determined by incubating them in 0.1 M sodium acetate buffer (pH 5.0) without substrate at 50, 60, and 70°C, taking samples after different time intervals and determining the glucoamylase activity as given in standard assay. Then the residual activities at each temperature were determined by taking the activity at 0 min as 100%.

Thermal deactivation kinetics of glucoamylase before and after cross-linking

Free enzyme, GA-CLEAs and pectin-CLEAs were incubated at 50, 60, and 70°C in 0.1 M sodium acetate buffer pH 5. The samples were withdrawn every 10 min for 60 min, chilled quickly, and then assayed for residual glucoamylase activity. A semi-log plot of percent residual activity versus time was plotted from which the inactivation rate constant, k_d, was calculated as the slope, and $t_{1/2}$, the time required for the activity to decrease to half its original activity was calculated as $0.693/k_d$. Further, the Arrhenius plot, i.e. plot of natural logarithm of k_d ($\ln k_d$) versus reciprocal of temperature in Kelvin scale (1/T) was obtained wherein slope of line indicates the deactivation energy (E_d). The difference in the deactivation energies (ΔE_d) of free enzyme, GA-CLEAs and pectin-CLEAs was calculated to quantify the thermal stability before and after cross-linking of the enzyme.

Determination of kinetic parameters

Kinetic parameters of free enzyme, pectin-CLEAs and GA-CLEAs were determined using different maltodextrin concentrations in the range of 0.2-3.0 mg/mL in 0.1 M sodium acetate buffer pH 5.0 at 50°C. In each form, equivalent amount of glucoamylase was used. K_m, V_{max} values of free enzyme, pectin-CLEAs and GA-CLEAs were calculated from non-linear

regression fitting of the initial reaction rates corresponding to different maltodextrin concentrations by Graph Pad Prism software.

Reusability of glucoamylase CLEAs

To evaluate the reusability of pectin-CLEAs and GA-CLEAs, pectin-CLEAs and GA-CLEAs were subjected to maltodextrin hydrolysis in batch mode under optimal conditions. After the specified reaction time, the pectin-CLEAs and GA-CLEAs were separated by centrifugation, washed twice with buffer and then suspended again in a fresh substrate to measure glucoamylase activity. The residual activity of glucoamylase was calculated by taking the glucoamylase activity of the first cycle as 100%.

Results and discussion

Preparation of pectin cross-linker

Pectin based cross-linker was prepared by controlled sodium metaperiodate oxidation of pectin. Controlled periodate oxidation of pectin results in partial oxidation of the hydroxyl groups on carbons 2 and 3 of the repetitive galacturonic unit. The partial oxidation of these groups leads to the rupture of bond between carbons 2 and 3 in the urinate residue, and to the formation of two aldehyde groups in each oxidized monomeric unit.[31] Formation of aldehyde groups in oxidized pectin was confirmed by FTIR analyses. Fig. 1 shows the FTIR spectrum of native and oxidized pectin. The significant feature observed in FTIR spectrum of oxidized pectin is appearance of new absorption peak at 1722 cm^{-1} which is corresponding to stretching vibrations of C=O in aldehyde group (Fig. 1b). This result is consistent with recent work on functionalization of pectin by periodate oxidation.[32, 27] Therefore, the FTIR analysis revealed that aldehyde groups have been successfully formed after periodate oxidation of pectin.

Preparation of glucoamylase pectin-CLEAs

Precipitation transforms the free enzyme into its aggregates form which is further captured in CLEAs through cross-linking. Due to the different biochemical and structural properties of enzymes, the nature of best precipitant which aggregates maximum enzyme activity varies from one enzyme to another.[6] Therefore, in order to capture maximum enzyme activity in CLEAs, it is necessary to screen a number of precipitants. Therefore we investigated seven protein precipitating agents: methanol, ethanol, iso-propanol, n-butanol, acetone, acetonitrile and ammonium sulphate for evaluating their abilities of precipitating glucoamylase. As shown in Fig. 2a, the precipitated activity of glucoamylase generated by ammonium sulphate was higher than that generated by organic solvents. Ammonium sulphate worked best, precipitating 98% soluble glucoamylase activity. So ammonium sulphate was selected as the optimized precipitating agent for further investigation.

The adding amount of ammonium sulphate also affected the amount of glucoamylase precipitated (Fig. 2b). The precipitated activity of glucoamylase was gradually increased with adding amount of saturated ammonium sulphate solution; however, the excessive adding of saturated ammonium sulphate solution resulted in the partial deactivation of the glucoamylase and the reduced precipitated activity of glucoamylase was observed. Similar deactivation of enzyme was observed due to addition of excessive ammonium sulphate during the preparation of esterase CLEAs.[33] The optimum adding amount of saturated ammonium sulphate solution was 10 mL for 2 mL glucoamylase solution (containing 530 U of glucoamylase and protein content 4 mg/mL) which precipitated 98% glucoamylase activity.

Cross-linking process can “lock” the enzyme into its active state and prevent redissolution (leaching) during reaction. The amount of cross-linker is important as it influences the

activity, stability, and particle size of the resulting CLEA.[34] Therefore the concentration of cross-linking agent must be optimized to recover maximum activity of enzymes in CLEAs. Oxidized pectin solution (40 mg/mL) prepared in 0.1 M acetate buffer of pH 5 was used as cross-linker for the preparation of pectin-CLEAs. The effect of concentration of cross-linker on the activity recovery of glucoamylase was studied using final cross-linker concentration in the range of 0.5-2.5 % (v/v). The variation of the oxidized pectin concentration modulated the extent of cross-linking by controlling the reaction between ε-amino groups of surface lysine or hydroxyl lysine side groups of the enzyme molecules. The activity recovery of glucoamylase in pectin-CLEAs increased when the oxidized pectin concentration increased. The highest activity recovered after pectin-CLEA production was 82.94% of the initial activity present in the free enzyme by using 1.5 %(v/v) of oxidized pectin solution (Fig. 3a). The higher oxidized pectin concentration lowered the activity recovery; this is the result of a higher rigidification of enzymes due to excessive cross-linking with the consecutive loss of the minimum flexibility needed for the activity of the enzyme. However, under low oxidized pectin concentration decrease in activity recovery of glucoamylase was observed. This could be due to insufficient cross-linking at lower oxidized pectin concentrations as glucoamylase activity was detected in supernatants collected after cross-linking using oxidized pectin concentrations less than 1.5 %(v/v). Therefore, the optimum oxidized pectin concentration for the maximum activity recovery of glucoamylase in pectin-CLEAs was 1.5 %(v/v).

In addition to amount of oxidized pectin, pectin cross-linking time also modulated the extent of cross-linking during preparation of pectin-CLEAs. It was observed that cross-linking occurred rapidly till 4 h cross-linking time as almost 50% activity recovery was obtained in pectin-CLEAs after 4 h cross-linking (Fig. 3b). Beyond 4 h, cross-linking continued but at a decreased rate as activity recovery was increased from 50% to 83% till 16 h cross-linking.

Cross-linking reactions with cross-linking time greater than 16 h resulted in a decrease in the activity recovery of glucoamylase in pectin-CLEAs but no glucoamylase activity was detected in supernatants collected after cross-linking times greater than 16 h. This result implied that prolonged pectin cross-linking time results in rigidification of enzyme due to a more intensive cross-linking which restricts enzyme flexibility abolishing enzyme activity.[35]

Preparation of glutaraldehyde-CLEAs (GA-CLEAs) of glucoamaylase

Usually, glutaraldehyde has been used as cross-linker for the preparation of CLEAs. The results of preparing glucoamylase-CLEAs with varying concentrations of the cross-linking agent glutaraldehyde are shown in Fig. 4. With the increasing of glutaraldehyde concentration, the activity recovery of glucoamylase in CLEAs was increased (Fig. 4a). When it was at 1.5% (v/v), 63.31% activity recovery of glucoamylase was achieved in CLEAs. Then, further increased the glutaraldehyde concentration, the activity recovery began to decline resulted from deformation of the enzyme tertiary structure because of more intensive cross-linking occurring at high glutaraldehyde concentration. Glucoamylase activity was detected in supernatants collected after cross-linking using glutaraldehyde concentrations less than 1.5 % (v/v) indicating insufficient cross linking. Subsequently, the optimal cross-linking time was investigated by using 1.5% (v/v) glutaraldehyde concentration. As shown in Fig. 4b, it can be seen that the activity recovery of glucoamylase in CLEAs increased at the beginning and then decreased with the prolonging of cross-linking time. After 16 h cross-linking, 64% activity recovery of glucoamylase was achieved in CLEAs. Incomplete cross-linking was observed at lower cross-linking time as glucoamylase activity was detected in supernatants collected after cross-linking times less than 16 h.

When using glutaraldehyde as cross-linker the activity recovery of glucoamylase in CLEAs was only 64 %. While when using pectin, the activity recovery of glucoamylase in CLEAs increased to 84%. Thus, glutaraldehyde-CLEAs showed an inferior activity to pectin-CLEAs.

Molecular secondary structure studies of pectin CLEAs by FTIR method

The catalytic activity of enzyme is highly related to the secondary structure of enzyme molecules. Changes in secondary structure of glucoamylase due to pectin cross-linking was analysed by FTIR. The FTIR spectra were acquired with free enzyme as well as pectin-CLEAs (Fig.S1 and Fig.S2). The most sensitive spectral region to the protein secondary structural components is the amide I band (1700-1600 cm^{-1}), which can be used to determine secondary structural components of the proteins.[36] Each type of secondary structural component gives rise to a somewhat different frequency in amide I band due to unique molecular geometry and hydrogen bonding pattern. However, due to the extensive overlap of the broad underlying component bands, which lie in close proximity to one another the secondary derivative FTIR analysis of amide I band was used to identify the component peaks' frequencies in order to improve the FTIR resolution.

The second derivative FTIR spectra of free enzyme and pectin-CLEAs were acquired (Fig. S3 and Fig. S4). The second derivative FTIR spectra of amide I region of free enzyme and pectin-CLEAs is shown in Fig.5. To obtain quantitative information about the protein secondary structure, a curve-fitting analysis of the second derivative spectra of amide I absorption band was performed to calculate the multicomponent peak areas. The areas of specific range in second derivative spectra correspond to specific types of secondary structure components and the fractions of each secondary structure could be obtained by these peak areas. Table 1 summarized the fractions of secondary structure for free enzyme and pectin-CLEAs samples. The glucoamylase in pectin-CLEAs showed a 12 % increase

in β- sheets as compare to free glucoamylase. This increase in β-sheets in pectin-CLEAs may be due to enzyme aggregates formation during pectin-CLEAs preparation which led to the form of intermolecular β-sheets.[37-38] On the other hand, 9% decrease in α-helix fraction, 11% increase in β-turn fraction and 14% decrease in random structure fraction was found upon pectin-CLEAs preparation. This means that glucoamylase molecules underwent significant changes on secondary structure upon pectin-CLEAs preparation and thus had activity loss of precipitated enzyme upon cross-linking.

Optimal conditions for glucoamylase activity

The glucoamylase activity of free enzyme increased gradually with temperature and a maximum activity was obtained at 50°C (Fig.6a). The optimum temperature for glucoamylase activity of the free enzyme was shifted to 55°C after pectin-CLEAs and GA-CLEAs formation. As was evident from the data, the glucoamylase in pectin-CLEAs and GA-CLEAs possessed temperature resistance than in free form. This shift in the optimum temperature can be explained by covalent bond formation between enzyme molecules caused by cross-linkers during CLEAs preparation. This formation might protect the enzymatic configuration from distortion or damage by heat exchange.[39-40]

As shown in Fig. 6b, highest activity of glucoamylase in both free enzyme and pectin-CLEAs was displayed at pH 5.0, whereas GA-CLEAs showed an optimal pH at 6.0. This means no change in optimum pH was observed after pectin-CLEAs formation. This result indicated that cross-linking with pectin did not change microenvironment around the active site of enzyme.

Thermal deactivation kinetics of glucoamylase before and after cross-linking

The temperature dependant loss of glucoamylase activity of free enzyme, GA-CLEAs and pectin-CLEAs is shown in Fig.7. From these temperature dependant activity profiles, the Arrhenius plot was obtained. The Arrhenius plot for free enzyme, GA-CLEAs and pectin-CLEAs is shown in Fig. 8. The slope of Arrhenius plot indicates the deactivation energy (E_d). The deactivation energy of glucoamylase in pectin-CLEAs, GA-CLEAs and free enzyme were found to be −7233.9 kJ $kmol^{-1}K^{-1}$, −6927.9 kJ $kmol^{-1}K^{-1}$ and −4906.5 kJ $kmol^{-1}K^{-1}$, respectively. When an enzyme is immobilized as cross-linked enzyme aggregates, a large number of covalent bonds are formed between individual enzyme molecules. These covalent bonds provide a more effective conformational stabilization of enzyme in CLEAs requiring much more energy to break down this active conformation than free enzyme. As a result, GA-CLEAs and pectin-CLEAs have higher deactivation energy than free enzyme.[11] However, compared to free enzyme the deactivation energy of glucoamylase in pectin-CLEAs was higher (ΔE_d = 2327.4 kJ $kmol^{-1}K^{-1}$) than in GA-CLEAs (ΔE_d = 2021.4 kJ $kmol^{-1}K^{-1}$). This indicates the excellent thermal stabilization of glucoamylase upon pectin-CLEAs formation. The half-life ($t_{1/2}$) and deactivation rate constants (k_d) of free enzyme, GA-CLEAs and pectin-CLEAs are given in Table 2. On an average, upon pectin cross-linking and glutaraldehyde cross-linking, there was 2.09 fold and 1.47 fold increase in the half-life of enzyme, respectively.

Kinetic parameters

Kinetic parameters of free enzyme, pectin-CLEAs and GA-CLEAs were determined by measuring initial reaction rates for each form with varying amounts of maltodextrin. As shown in Table 3, K_m values of free enzyme and pectin-CLEAs are nearly equal whereas K_m value of GA-CLEAs is increased. This indicated the enzyme affinity for the substrate

remained unchanged after pectin-CLEAs formation and decreased after GA-CLEAs formation. Moreover, no change in K_m values also suggests that pectin-CLEAs do not limit the permeation rate of substrate and product. This could be possibly explained on the basis that compared to compact super-molecular structure formed by micro-molecular glutaraldehyde cross-linker, macromolecular pectin as a cross-linker enlarges structure of pectin-CLEAs due to bonding of glucoamylase along its long chain which prevents the lump of enzyme molecules resulting in improved internal mass transfer of substrate and product.[21]

Similarly, V_{max} values of free enzyme and pectin-CLEAs are nearly equal whereas V_{max} value of GA-CLEAs is decreased which indicated the rate of maltodextrin hydrolysis was not changed after pectin-CLEAs preparation. This suggested that the flexibility of glucoamylase was maintained even after cross-linking of enzyme molecules in pectin-CLEAs than in GA-CLEAs. It could be due to bonding of glucoamylase molecules along long chain of macromolecular pectin as a cross-linker which prevented restriction of enzyme molecules in a finite district with weak flexibility.

Reusability of glucoamylase CLEAs

Reusability of immobilized enzyme preparation is important for its cost-effective industrial application. The reusability of the glucoamylase in pectin-CLEAs and GA-CLEAs was examined for repeat applications in a batch reactor. Glucoamylase activity retention of the pectin-CLEAs and GA-CLEAs on repeated use is shown in Fig. 9. Glucoamylase activity decreased while reuse number is increased. The results show that after the 10 cycles of use pectin-CLEAs retained about 55% of its initial activity, whereas GA-CLEAs retained only 29% of its initial activity. According to these results pectin cross-linked glucoamylase CLEAs have potential use in industrial applications.

Conclusions

The results presented in this study demonstrate preparation of glucoamylase pectin-CLEAs by using pectin as the cross-linking agent. Pectin was oxidized with sodium metaperiodate to generate aldehyde groups and used as cross-linker. It was demonstrated that the type of precipitant, amount of precipitant, amount of oxidized pectin and cross-linking time had significant effects on the glucoamylase activity recovery in pectin-CLEAs. Compared with traditional glutaraldehyde, glucoamylase CLEAs cross-linked with pectin showed excellent activity recovery. Analysis of the secondary structure showed significant change in secondary structure of glucoamylase upon pectin-CLEAs formation. Glucoamylase in pectin-CLEAs was significantly more stable than free glucoamylase and glucoamylase glutaraldehyde-CLEAs when incubated at 50-70°C. Glucoamylase rate of maltodextrin hydrolysis and affinity towards maltodextrin remained same after pectin-CLEAs formation and decreased after glutaraldehyde-CLEAs formation. Additionally, pectin-CLEAs could still retain 55% of their original activity after successive re-use for 10 batches compared to only 29% of their original activity retained by traditional glutaraldehyde-CLEAs. These results highlight the potential use of pectin as the cross-linking agent for the formation of pectin-CLEAs. As pectin is abundant, renewable and biocompatible natural biopolymer, its use as cross-linking agent has considerable advantages from environmental and worker safety points of view over the traditional chemicals traditionally used.

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Notes and References

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Figure captions

Fig.1 FTIR spectra of (a) native pectin (b) oxidized pectin.

Fig.2 (a) Precipitation of glucoamylase with different precipitants (b) Effect of adding amount of saturated ammonium sulphate solution on precipitated activity of glucoamylase. The 98% precipitated activity corresponds to 519.4 U of glucoamylase.

Fig.3 Effect of (a) oxidized pectin concentration (b) cross-linking time on activity recovery of glucoamylase in pectin-CLEAs. The 83% activity recovery corresponds to 55 U/mg aggregate (total 440 U) of glucoamylase. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Fig.4 Effect of (a) glutaraldehyde concentration (b) cross-linking time on activity recovery of glucoamylase in GA-CLEAs. The 64% activity recovery corresponds to 42.40 U/mg aggregate (total 339.2 U) of glucoamylase. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Fig. 5 Second derivative amide I spectra of (a) free enzyme (b) pectin-CLEAs.

Fig. 6 Influence of temperature (a) and pH (b) on glucoamylase activity of free enzyme (♦), GA-CLEAs (■) and pectin-CLEAs (▲). In each case assuming the highest glucoamylase

activity of free enzyme, GA-CLEAs and pectin-CLEAs as 100%, respectively. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Fig. 7 Thermal deactivation of (a) free enzyme (b) GA-CLEAs and (c) pectin-CLEAs at 50°C (♦), 60°C (■) and 70°C (▲).

Fig. 8 Arrhenius plot for free enzyme (♦), GA-CLEAs (■) and pectin-CLEAs (▲).

Fig.9 Reusability of glucoamylase in pectin-CLEAs and GA-CLEAs. The 100% residual activity of glucoamylase in first cycle corresponds to 55 U/mg aggregate for pectin-CLEAs and 42.40 U/mg aggregate for GA-CLEAs. The experiments were done in triplicate and the error bar represents the percentage error in each set of readings.

Table 1 Fractions of secondary structures for free enzyme and pectin-CLEAs

Form of enzyme	α-helix	β-sheets	β-turn	random structure
Free enzyme	27.9	30.3	22.6	19.2
Pectin-CLEAs	18.7	42.5	33.4	5.4

Table 2 Thermal deactivation coefficient (K_d) and half-life ($t_{1/2}$) of free enzyme, GA-CLEAs and pectin-CLEAs

Temperature (°C)	K_d (min^{-1})			$t_{1/2}$(min)			Fold increase in $t_{1/2}$	
	Free enzyme	GA-CLEAs	Pectin-CLEAs	Free enzyme	GA-CLEAs	Pectin-CLEAs	GA-CLEAs	Pectin-CLEAs
50	0.0386	0.0197	0.0134	17.95	35.17	51.71	1.95	2.88
60	0.042	0.0377	0.0279	16.5	18.38	24.83	1.11	1.50
70	0.0935	0.0688	0.0494	7.41	10.07	14.028	1.35	1.89
Average of fold increase in the half-life over the range of 50-70°C							1.47	2.09

Table 3 Kinetic parameters of free enzyme, pectin-CLEAs and GA-CLEAs

Form of enzyme	K_m (mg/mL)	V_{max} (µmol/min)
Free enzyme	0.63 ± 0.024	6.22 ± 0.12
Pectin-CLEAs	0.64 ± 0.017	6.20 ± 0.29
GA-CLEAs	1.16 ± 0.089	4.17 ± 0.34

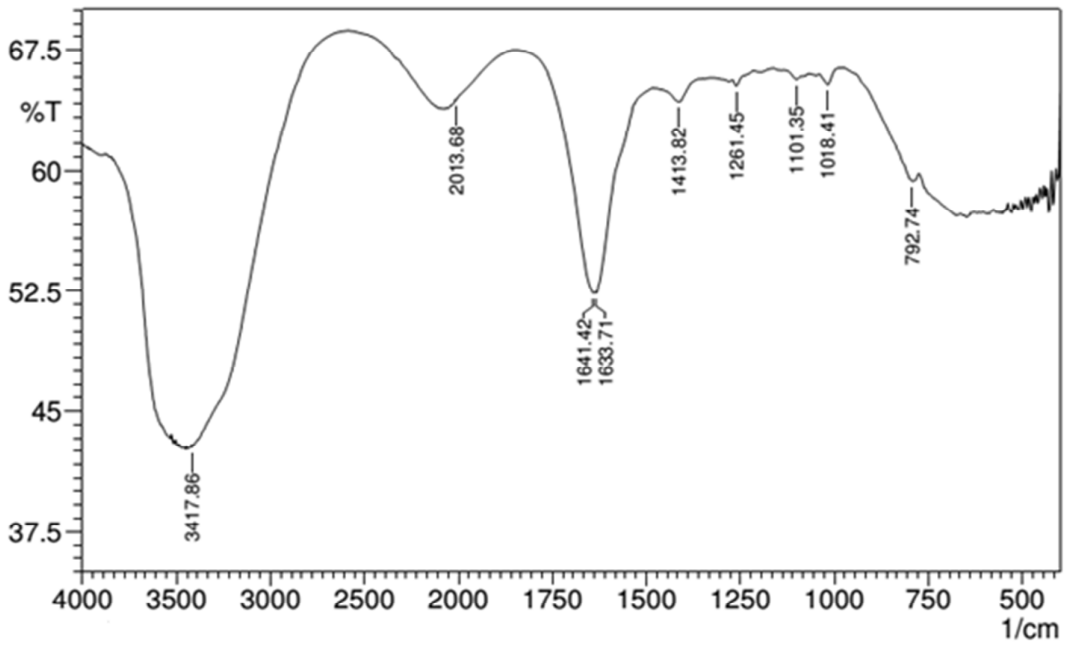
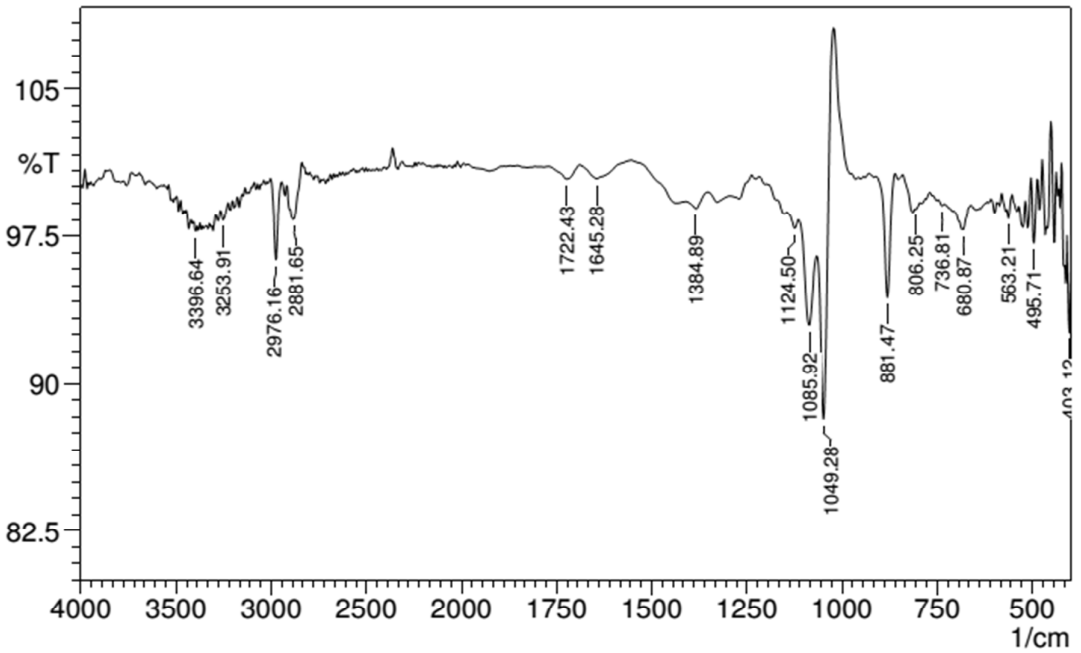


Fig. 1a



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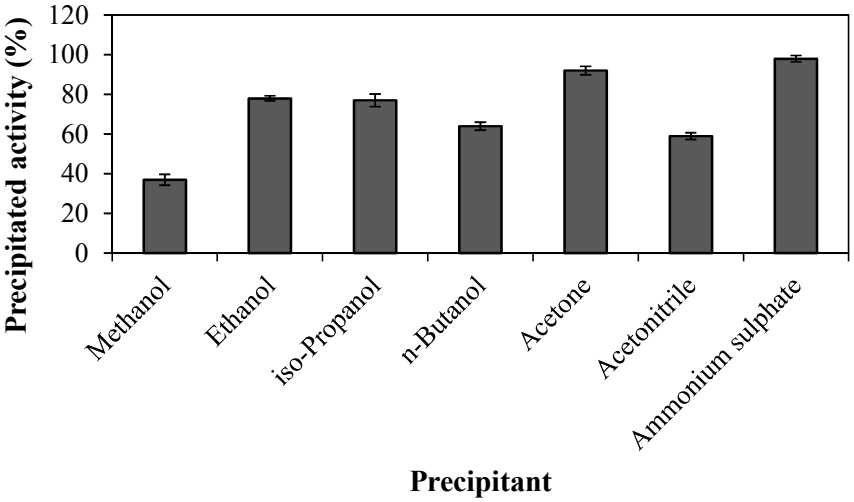
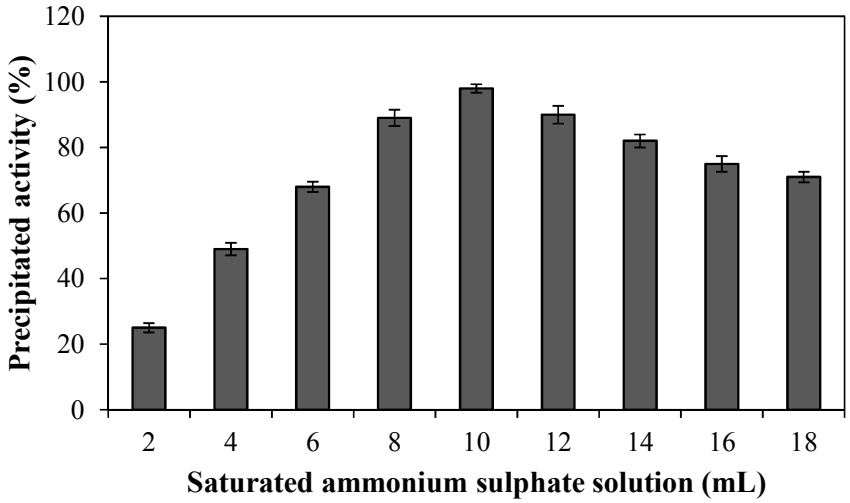


Fig 2a



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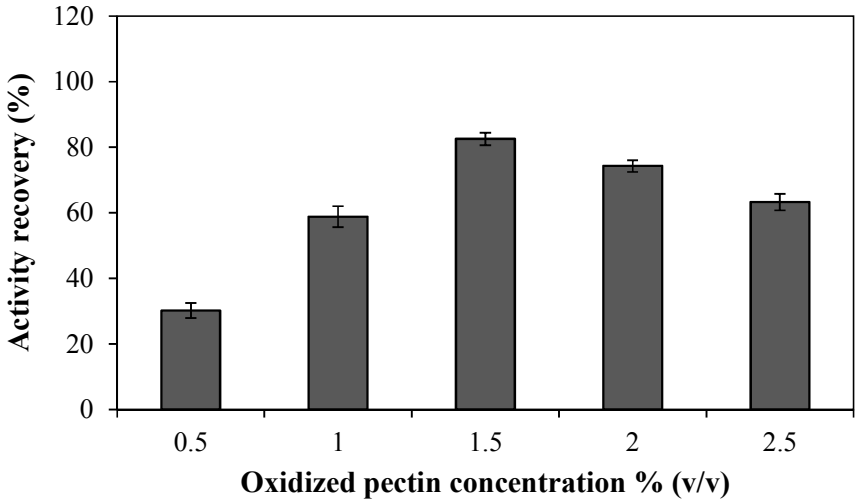

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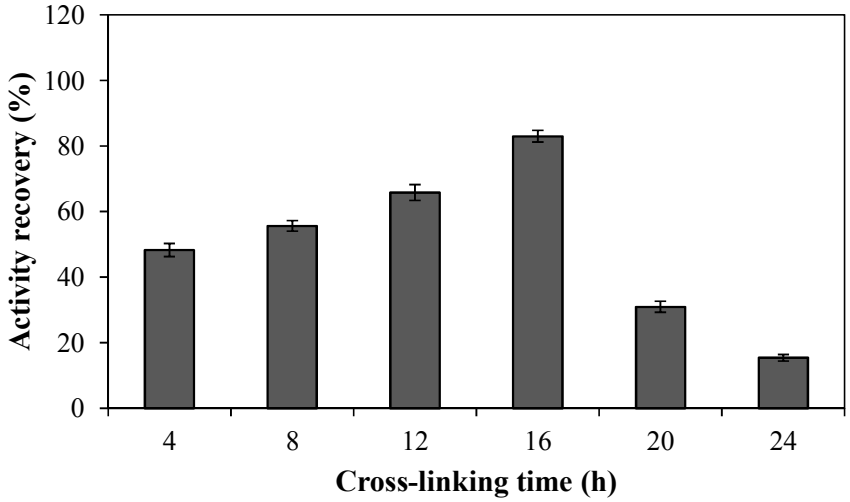

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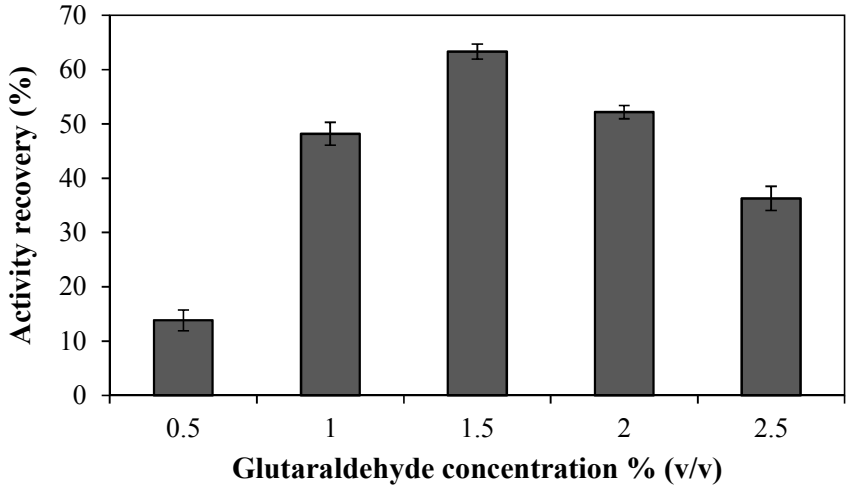

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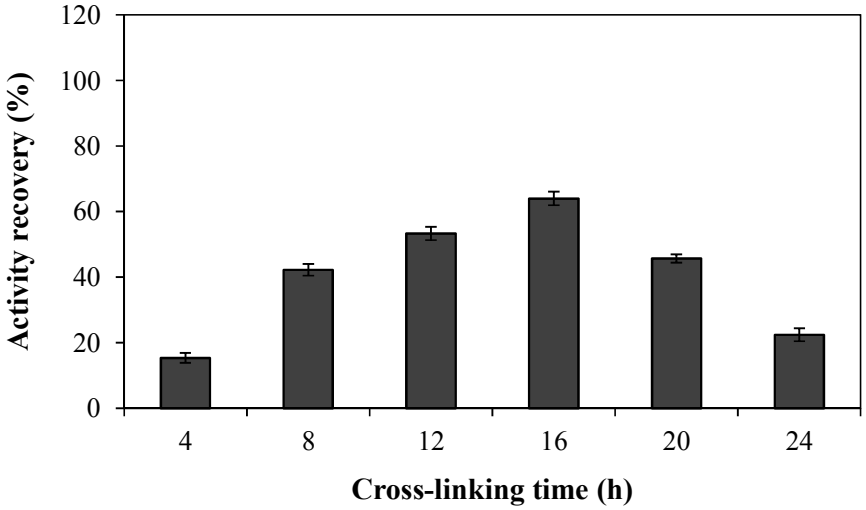
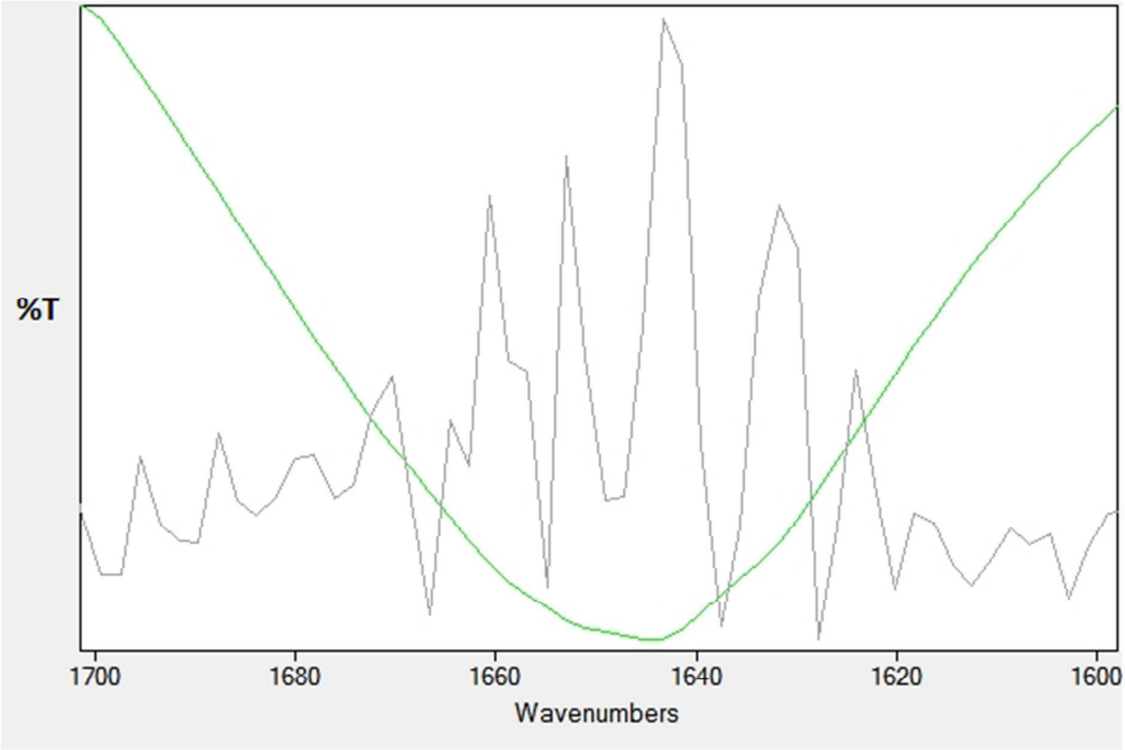


Fig.4b



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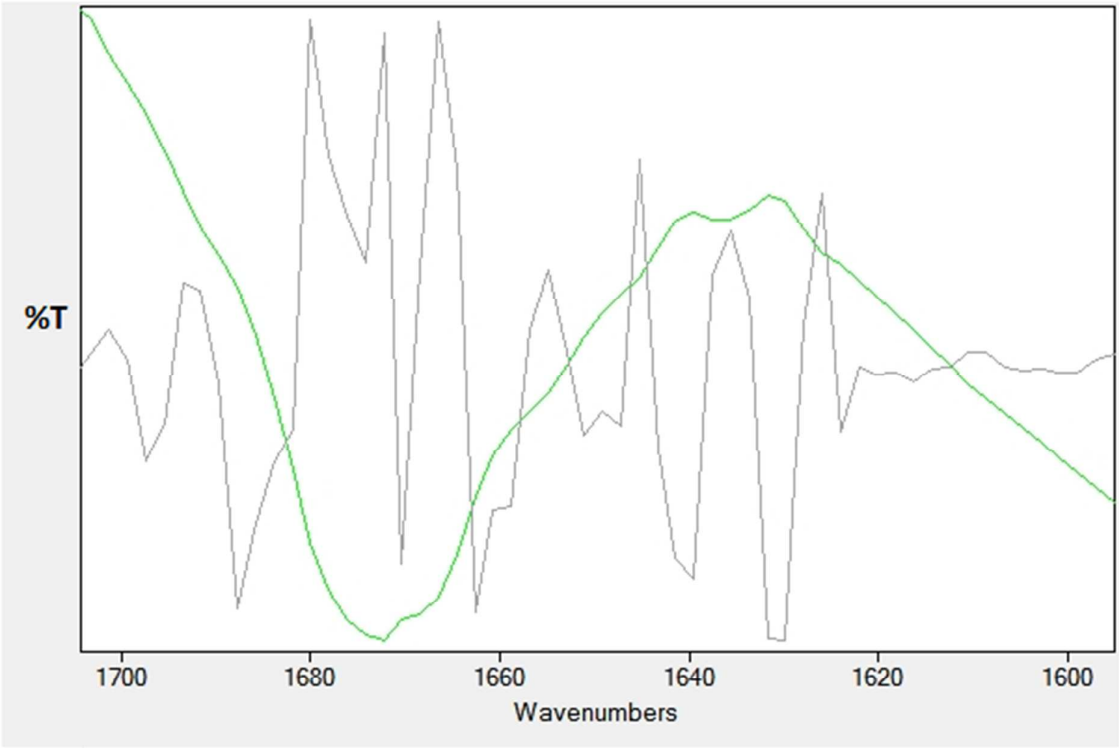

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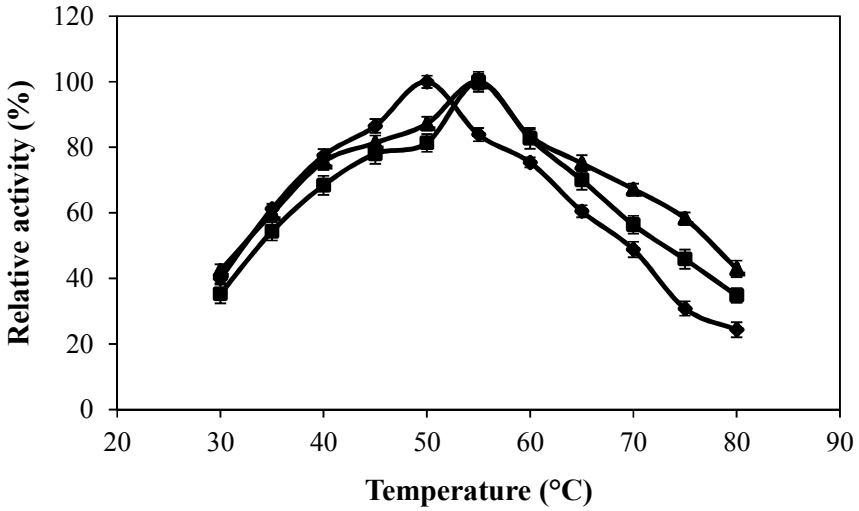

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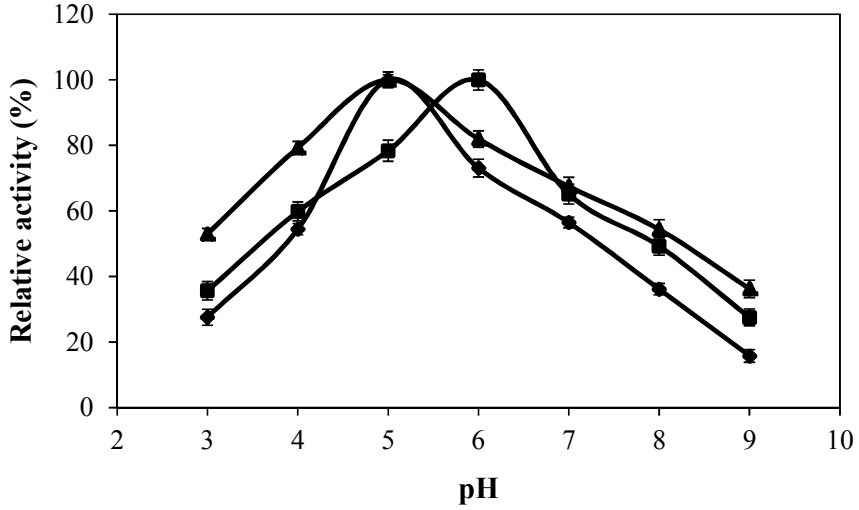

Fig. 6b

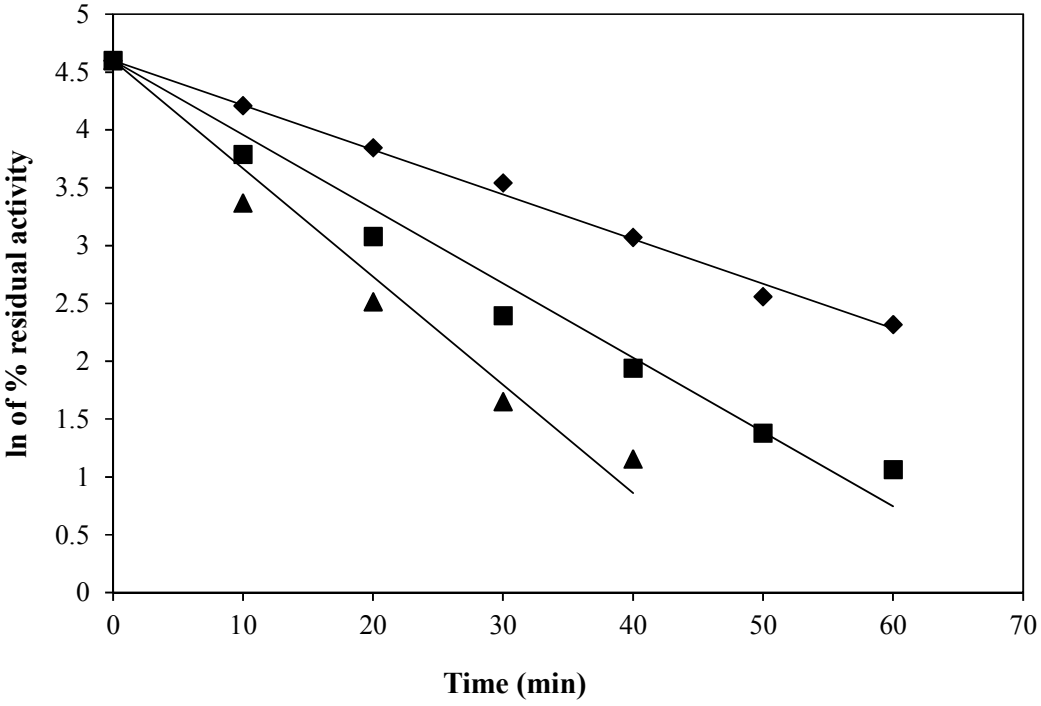

Fig. 7a

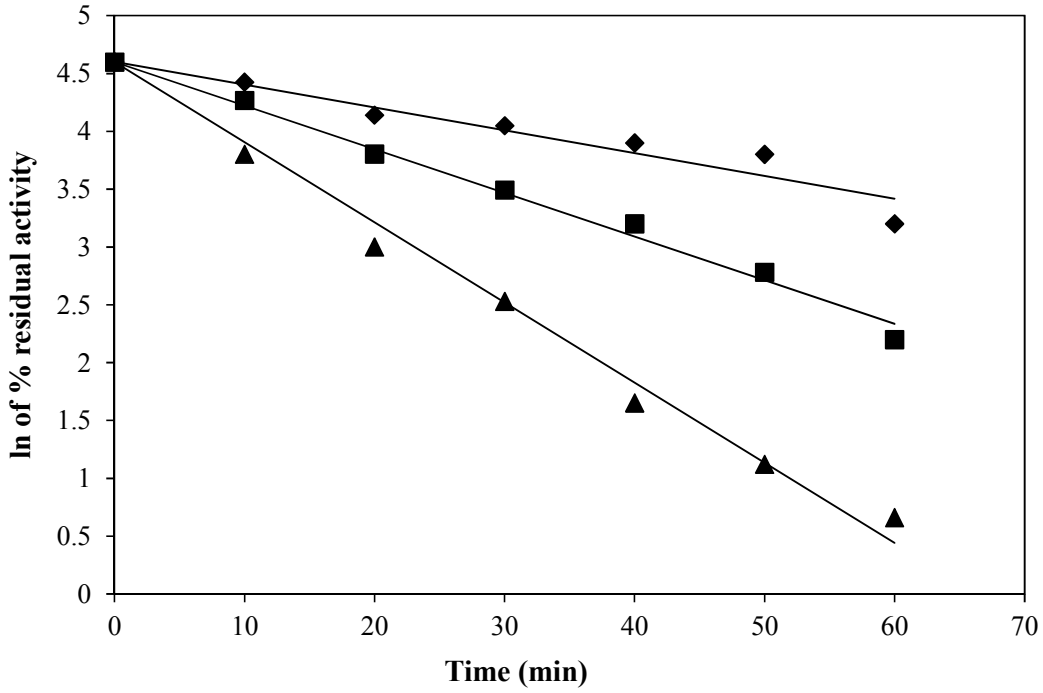

Fig. 7b

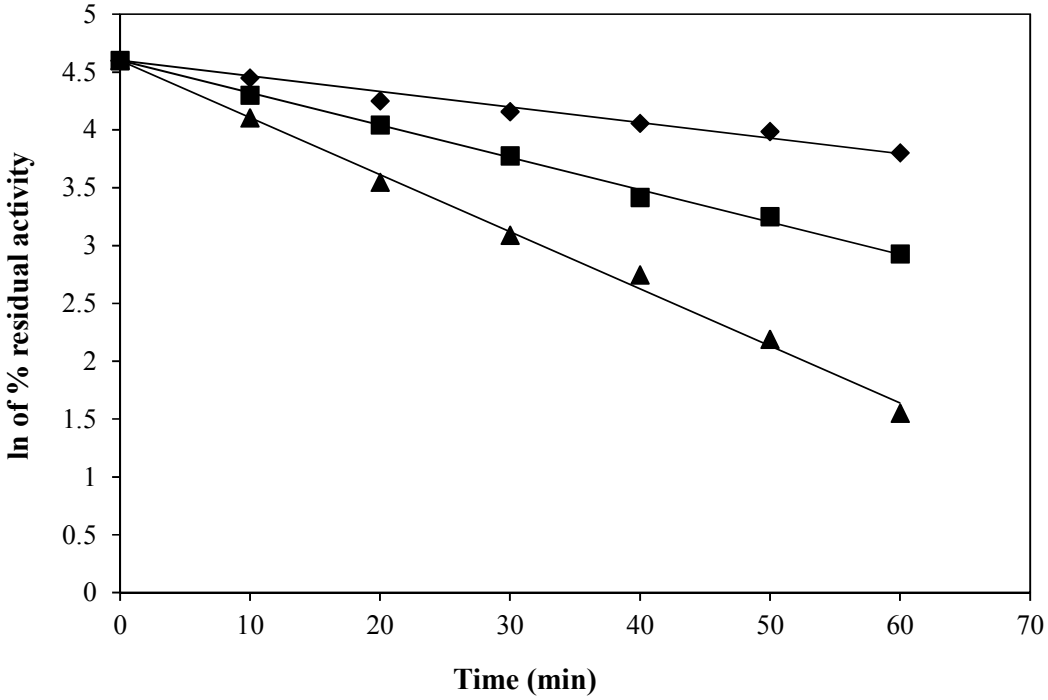

Fig. 7c

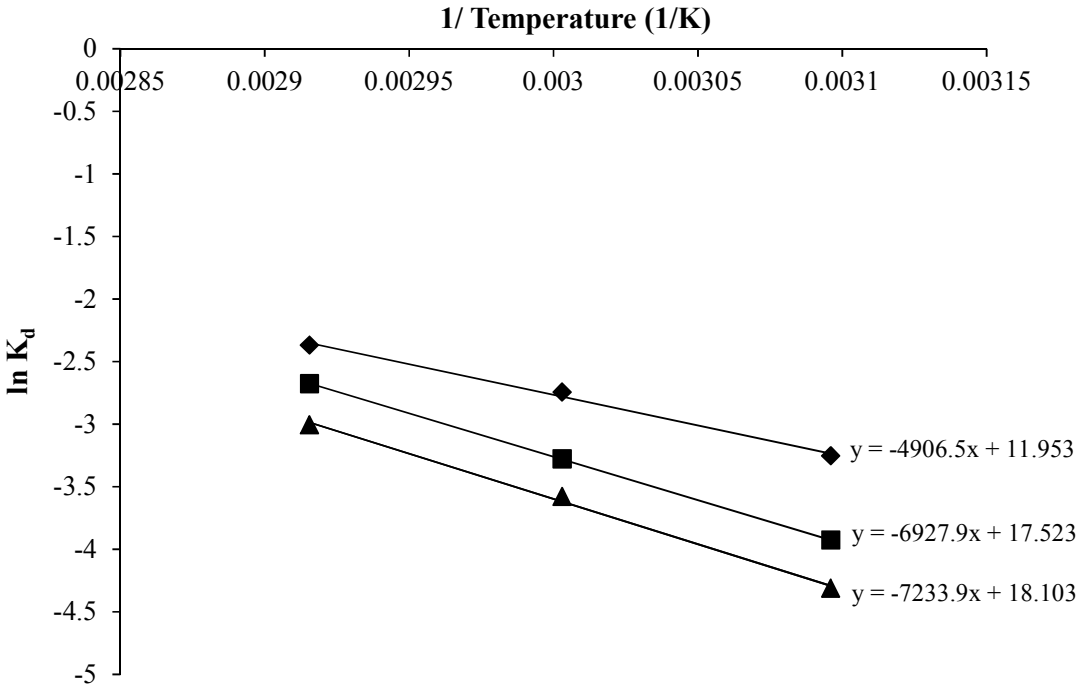

Fig. 8

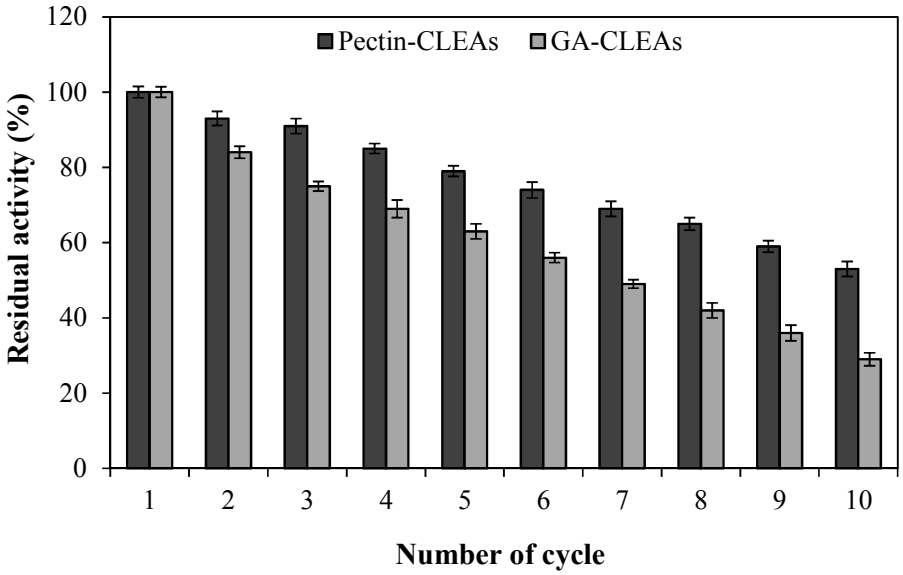

Fig.9