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Cellulase stabilization by crosslinking with ethylene glycol dimethacrylate and evaluation of its activity including in water-ionic liquid mixture

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Abstract

Synthesis of immobilized enzymes via crosslinking is an easy route to develop biocatalyst with enhanced activity and recyclability. In the present study, cellulase from *Aspergillus niger* was crosslinked by ethylene glycol dimethacrylate (EGDMA) using ammonium persulphate (APS) as a initiator to obtain heat and pH stable crosslinked cellulase aggregates (CCAs) using 5% EGDMA and 1% APS for crosslinking. The pristine cellulase and the CCAs were characterized by various techniques. Their activity profiles were evaluated as a function of time, temperature and pH. The activity of the CCAs was further evaluated in 1–butyl–3–methyl imidazolium acetate [bmim][Ac] at the optimized conditions of time and temperature. Furthermore, the CCAs were reusable upto 12 repeat cycles with retention of 58% of its initial activity after 7th cycle in the hydrolysis of cellulose. Artificial neuron network (ANN) model was employed to correlate the relationship between process parameters and the %relative activity (RA). The predicted %RA values are in close agreement with the experimental values.

Introduction

Enzyme stabilization by carrier bound immobilization methods such as adsorption,[1] encapsulation,[2] and covalent binding[3] are relatively tedious and time consuming protocols due to the operational complications.[4] Contrary to these approaches the carrier-free approach i.e. crosslinking of enzymes to crosslinked enzyme aggregates (CEAs) is a promising method

for immobilization of enzymes.[5] This approach involves low production cost and simple protocol. There is unusual advantage of high concentration of enzyme active sites over a small volume due to the negligible molecular weight of the crosslinker.[6] One CEA can contain thousands of enzyme molecules.[6b] Exclusion of enzyme carrier, which is a non–catalytic mass, results in the enhancement of catalytic activity.[7] Thus, this synthetic protocol offers advantages of high enzyme activity and simplicity of crosslinking, besides retaining all the advantages of the carrier–based immobilization methods such as reusability, recyclability, operational stability over wide ranges of temperature and pH[8] and high thermal and storage stability.[9] Therefore, several enzymes such as peroxidase,[7a] lipase,[7b,10,] penicillin acylase,[11] invertase,[12] penicillin G amidase,[13] α–amylase,[14] etc., have been successfully stabilized by crosslinking with high activity retention. The CEAs have been used in a number of biocatalytic reactions such as synthesis of ampicillin,[11] alkyl ferulates,[15] β–lactam antibiotics,[16] resolution of *N*–(2–Ethyl–6–methylphenyl) alanine[17] and elimination of endocrine disruptors.[18]

The general protocol of CEA synthesis involves protein precipitation followed by crosslinking with the multifunctional reagents.[19] Glutaraldehyde is the most commonly reported crosslinker in enzyme crosslinking as its aldehydic groups react with surface amino groups of amino acids of enzyme to form a crosslinked network. However, due to its small size, it diffuses into the inner part of the enzyme and thus restricts accessibility to the macromolecular substrates.[20] The crosslinking reaction can also involve some amino acids of active sites leading to the loss of enzyme activity.[21] Glutaraldehyde crosslinked enzymes have other drawbacks of low activity retention, poor mechanical stability and reproducibility.[6b] Furthermore, glutaraldehyde is toxic and non–usable in biomedical applications. Therefore, the use of macromolecular crosslinking reagents is an alternative that needs to be investigated to avoid the diffusional restrictions of CEAs and impart more stability to the enzyme. In view of this, dialdehyde starch and dextran polyaldehyde have been successfully employed to synthesize CEAs via the Schiff base formation.[22]

In our previous work, *N,N*-methylene bisacrylamide was successfully used as a macromolecular crosslinker to generate CEAs.[23] In the present work, a different crosslinker, ethylene glycol dimethacrylate (EGDMA) is introduced to crosslink cellulase. It can be predicted that CEAs of cellulase, hence after referred to as CCAs, prepared using glutaraldehyde would exhibit low activity towards hydrolysis of cellulose which itself is a macromolecular substrate. In view of this, EGDMA was used as an alternative to avoid all the limitations of glutaraldehyde. The parametric studies were carried out of the pristine

cellulase and the CCAs with respect to time, temperature and pH and also evaluated for their thermal and storage stability. The reusability studies of CCAs were carried out for cellulose hydrolysis upto 12 repeat cycles. The cellulose hydrolysis catalyzed by the CCAs in 1–butyl–3–methyl imidazolium acetate [bmim][Ac] was explored thus proving its stability in ionic liquids also.

Results and discussion

Synthesis and characterization of CCAs

Aspergillus niger cellulase was crosslinked by thermally initiated free radical crosslinking using ammonium persulphate (APS) as initiator[23] and EGDMA as crosslinker. The crosslinking reaction proceeds by free radical polymerization reaction. Ammonium persulphate (APS) generate free radicals, which initiate polymerization of EGDMA. The free radicals thus generated further interact with enzyme to generate crosslinked enzyme aggregate. The CCAs were characterized by Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), particle size analysis and X–ray diffraction (XRD) to get evidence for crosslinking. In FTIR spectra, appearance of peaks at 1647.3 cm^{-1} and 1422.5 cm^{-1} for pristine and 1642.5 cm^{-1} and 1456 cm^{-1} for CCAs confirms the presence of cellulase in samples (**Fig. 1**). In literature, the characteristic peaks of cellulase at 1624 cm^{-1} and 1408 cm^{-1} have been reported[24] which supports the precise spectra of present study. In addition to the above peaks, FTIR spectrum of the CCAs (b) has a prominent band at 1728.6 cm^{-1} that is assigned to the –C=O stretching and at 1255 cm^{-1} can be assigned to C–O–C stretching of crosslinker EGDMA and 1154 cm^{-1} due to the C–N stretching vibration. In the FTIR spectrum of pristine cellulase (a) the peaks at 853.45 cm^{-1} and 909 cm^{-1} are due to –NH out of plane bending (wagging and twisting) of amino groups, which are disappeared in spectrum of CCAs. It is revealed that $–NH_2$ groups of cellulase are also the possible crosslinking sites so these may be consumed in the crosslinking reactions.[23]

Surface morphology of the CCAs was characterized from SEM images at different magnifications (**Fig. 2**). It appears therefrom that the crosslinking resulted in nanoparticle formation as small spheres can be seen on the surface of the CCAs. The CCAs are aggregated particles of spherical shape and have porous appearance. Similar surface morphology has been reported elsewhere for glutaraldehyde crosslinked lipase.[6b] The CCAs particles are spherical nature as is also evident from the TEM images. Each enzyme particle is ~50 nm

size. It appears therefrom that crosslinking resulted in nanoparticle formation (**Fig. 3**). The TEM image is result of crosslinking of many enzyme molecules together. Such crosslinking can be expected with a crosslinker like EGDMA where chain reaction can take place to bring together thousands of enzyme molecules. It has been reported in literature even that crosslinking with glutaraldehyde brings together one aggregate up to thousands of lipase molecules.[6b] Particle size analysis of the pristine cellulase and the CCAs reveals an interesting aspect of this study. Both show almost very narrow size distribution and the same size of ~1 μm. It appears that swelling in the 1,4 dioxane and water and for pristine cellulase and CCAs, respectively, makes these to have higher size than the actual (**Fig. S1 supplementary material**). Such a uniform morphology of the CCAs with nano–size has not been reported in literature. Contrary to these results, enzyme aggregates with wide size distribution, from 1 up to 100 μm size, have been reported.[25] XRD pattern of the pristine cellulase exhibits more intense peaks as compared to the peaks in the XRD pattern of the CCAs, which is typical for its semi-crystalline structure (**Fig. S2 supplementary material**). The XRD pattern of the CCAs displays peaks of low intensity indicating a predominantly amorphous structure of cellulase. The disappearance of crystalline peaks due to decrease in the intensity in XRD pattern of CCAs indicates that crosslinking induces amorphous nature to the cellulase structure.

Activity analysis of pristine cellulase and CCAs

Enzyme activity of the pristine cellulase and the CCAs was determined using 3,5–dinitrosalicylic acid (DNS) method.[26] The results are provided in relative form with the highest value being 100 % activity. Relative activities of the pristine cellulase and the CCAs were calculated from the relationship – Relative activity (%) = activity of pristine cellulase or CCAs/ highest activity × 100. The relative activities were obtained with respect to time, temperature and pH (**Fig. 4**). Activity of the pristine cellulase as well as the CCAs increased with contact time. Optimum activity was obtained at 20 min for the pristine cellulase and at 30 min for the CCAs. The contact time for the CCAs is higher as being a polymeric network it takes time to expand fully and expose the reactive sites to the substrate. Activity profile of the CCAs shows better results than the pristine cellulase throughout the range of temperature studied. Thus, demonstrating that crosslinking imparts thermal stability or thermophilicity to cellulase. Optimum temperature for the pristine cellulase was 55 ºC whereas for CCAs it was 65 ºC. The CCAs retain high activity even at temperature higher than the optimum. Even for the pre-treated cellulose the cellulase activity has been reported to be the maximum at 60 ºC.[27] Optimum pH was found to be 5.6 for both the pristine as well as the CCAs. Beyond the

optimum pH, the activity of the pristine cellulase decreased drastically, and in contrast the decrease in the case of CCAs was much slower implying stability under the harsh chemical environment. In literature pH stability over a narrow range, but high thermal stability has been reported.[28] For the sake of comparison only two parameters, variation of temperature and pH, were chosen for comparison as the other parameter, contact time, was studied at different ranges. %Relative activities of the crosslinked enzymes (CCAs and crosslinked lipase aggregates (CLLAs[23]) have been calculated at different temperatures and pH in comparison to their pristine forms and are presented in Table 1. Therefrom it is obvious that the performance of new CCAs generated with the crosslinking agent EGDMA is far better than the earlier reported *N,N*-methylene bisacrylamide (*NN*MBAAm)-crosslinked CLLAs.[23] In the former, post crosslinking changes resulted in the enhancement of the enzyme activity with respect to their pristine form while in the latter case it decreased.

Thermal and storage stability of pristine cellulase and CCAs and reusability of CCAs

Thermal stability of the pristine and the CCAs was obtained at 60 ºC which revealed better results for the CCAs thus making it suitable candidate for industrial application (**Fig. 5**). At 60 ºC, the CCAs retained 91.240% of its initial activity after 100 min while pristine cellulase retained only 66.267% of its initial activity. Storage stability of the CCAs was also better than the pristine one retaining 61.246% of its initial activity after 30 days whereas pristine cellulase retained only 37.991% (**Fig. 6**). The CCAs are easily recyclable and being insoluble can be used in multiple cycles at optimum conditions of 30 min. and 65 ºC (**Fig. 7**). The CCAs retained 58% of its initial activity after 7 cycles for hydrolysis of cellulose indicating an adequate operational stability of the CCAs and making it suitable candidate for the cost effective industrial use. Such improvement in the activity of the CCAs is result of high concentration of active sites in the nano–form which makes maximum contact with the substrate. Also, post–crosslinking conformational changes in CCAs favour higher activity than the pristine cellulase.

Activity of CCAs in 1–butyl–3–methyl imidazolium acetate [bmim] [Ac]

Activity of CCAs was also studied in 1–butyl–3–methyl imidazolium acetate [bmim][Ac] at the optimum conditions i.e. at 30 min. and 65 ºC (**Fig. 8**). The activity of the CCAs was found to be the maximum when the ratio of water and ionic liquid was 1:0.75,

higher than what it was without [bmim][Ac] other conditions being the same. It can be inferred therefrom that within a short contact time the ionic liquid decreases crystallinity of cellulose and opens up the cellulose structure resulting in the enhanced cellulose hydrolysis. However, it decreased with an increase in the amount of ionic liquid. Even a small trace of ionic liquid has been reported to adversely affect cellulase activity.[26] However; in the present case we obtained better hydrolysis result in the presence of ionic liquid. The rate of hydrolysis beyond 1:0.75 (water: ionic liquid) did not necessarily decrease due to the denaturation or adverse effect of [bmim][Ac], rather the amount of water also matters.

Application of ANN model to evaluate %RA

Artificial neural network (ANN) model was developed to estimate the %RA as a function of time, temperature and pH using the experimental parametric framework. The statistical results of the inputs and output parameters are presented (**Fig. S3 supplementary material**). The variable normalization process was used with respect to the maximum and the minimum values in the entire data set. The ANN model was trained using a back propagation algorithm and the sigmoid function was used as an activation function. A detailed description of the back propagation algorithm and training procedure has been reported in literature.[29] The ANN model consisted of three neurons (time, temperature and pH) in the input layer and one neuron (%RA) in the output layer. The ANN model training involves of adjusting the weights associated with each connection between the neurons until the computed outputs for each set of input data are as close as possible to the experimental output values. Total available experimental data sets were 18. The developed ANN model can be used to predict and analyze %RA through performing sensitivity analysis at various process parameters. The sensitivities provide the first approximation of the effect of various perturbations, and thus provide an initial assessment as to which interventions might be the most useful. In the present work, the sensitivity analysis was calculated by changing one input parameter at small intervals while keeping the other elements unchanged and estimating the output, and changing the two input parameters and studying their effects on the output. The obtained results based on the above model were compared with the experimental %RA. It is evident there from that there is only a small variation between the predicted and the experimental values. The average %error for the CCAs and the pristine cellulase were found to be 1.875 and 0.94, respectively. Very high values of R^2 = 0.99957 and 0.99961 were obtained for training and testing data set. The interactions between different parameters significantly influenced % RA even with a small variation of the input parameter.

Fig. S4 (**supplementary material**) represents the processing window for optimum %RA with the variation of time and temperature at different pH. It can be observed therefrom that the CCAs are active even at low temperature, but at longer time intervals than the optimum value. At pH 5.6, the highest %RA can be obtained over a wide range of time and temperature, i.e. at a short interval of time, but on both sides of the optimum temperature. In conclusion, the results reported have been supported by the ANN model, yet the use of the operational window can be useful for determining the input parameters in future studies.

Conclusions

We have successfully demonstrated a simple and green crosslinking protocol that enhanced cellulase activity. The CCAs is thermophillic and stable at a wide spectrum of pH. It also has good storability, recyclability and reusability when studied up to 12 cycles. The obtained ANN model predictions are close with the experimental % RA values. The CCAs is also active in the medium having ionic liquid. All the reported features of the CCAs made this crosslinking method technologically acceptable as it involves non–toxic crosslinker, simple, low cost and green protocol.

Experimental

Materials

Potassium dihydrogen phosphate, Disodium hydrogen phosphate, cellulose (S.D. fine chem. Limited, Mumbai, India), *Aspergillus niger* cellulase, 1 butyl 3 methyl imidazolium acetate (Sigma Aldrich) Glucose, Sodium hydroxide, DNS (3,5–Dinitrosalicylic acid) (HIMEDIA), Ammonium persulphate, Sisco Research Laboratory Pvt. Ltd. Bombay, India), Ethylene glycol dimethacrylate (Merck, India), were used as received.

Synthesis of CCAs

Aspergillus niger cellulase was dissolved in distilled water and its crosslinking was achieved using EGDMA as crosslinker. 5% EGDMA and 1% APS was added to cellulase solution and mixture was kept at 65 ºC for 5h in order to allow its crosslinking. The solid CCAs thus obtained was separated by simple filtration, washed with water and dried in air.

Characterization of CCAs

CCAs were characterized by Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), particle size analyzer and X–ray diffraction (XRD) to get evidence of crosslinking. FTIR spectrum was

recorded on Perkin Elmer in transmittance mode in KBr. FE-SEM and TEM images were recorded on JEOL JSM-7000F and JEM2010 JEOL, respectively. Particle size measurements were obtained from Nanowave particle size analyzer (Metrohm). X–ray diffraction patterns of samples were recorded on Riago Smart Lab 9 Kv diffractrometer system using a typical wavelength of 1–54060 Å (Cu– Kα radiation). The diffraction angle 2θ was varied from 10 to 70 degrees.

Activity assay

Activity of the pristine cellulase and the CCAs was determined spectrophotometrically by carrying out the hydrolysis of cellulose.[30] 1% (w/v) solution of cellulose was prepared in phosphate buffer of pH 5.5. The reaction mixture comprised of stock solution and 10 µL pristine or 10 mg CCAs and to make final volume upto 3 mL. The reaction mixture was incubated at 50 °C for 15 min. 1 mL of DNS reagent was added to this reaction mixture and kept in boiling water bath for 15 minutes. Reaction mixture was cooled at room temperature and read at 540 nm against blank prepared from 1.0 mL of DNS reagent and 2.0 mL of phosphate buffer. A calibration curve of the absorbance and known glucose concentrations was plotted. Calculations were made by using commercial glucose as standard. Enzyme activity was studied as a function of time (10 to 50 min. after 10 min. interval), temperature (25 to 65 ºC after 10 ºC interval) and pH (1.2, 4.0, 5.6, 7.0, 8.5 or 9.0). All the experiments were carried out in duplicate and reported values are average of the two values. The reusability studies of the CCAs were carried out for twelve repeat cycles at the optimized conditions of time, temperature and pH.

Thermal and storage stability

For obtaining thermal stability of the pristine cellulase and the CCAs, their samples were incubated at 60 ºC. After every 20 min, 10 µL of the pristine or 10 mg of the CCAs was taken out from incubator and added to reaction mixture. The activity of enzymes was obtained up to100 min. by using the earlier discussed method. Calculated optimum activity of the pristine and the CCAs was taken as 100% and then both enzymes were stored at 4 ºC and activity was obtained after every 10 days upto 30 days using the same procedure to find out its storage stability.

Evaluation of CCAs activity in ionic liquid

For the evaluation of activity of CCAs in ionic liquid, different volume ratio of the water to ionic liquid (1:0 to 1:5) were prepared and stock solution of cellulose was added to mixtures, and final concentrations were made up to 1%. The CCAs activity was obtained

using the method described earlier at the optimized conditions of time and temperature i.e. at 30 min. and 65 ºC.

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Table 1

Temperature (ºC)	CCAs	CLLAs[23]	pH	CCAs	CLLAs[23]
25	192.22	58.75	1.2	153.11	42.51
35	148.20	63.49	4.0	119.49	62.18
45	162.89	77.56	5.6	141.91	73.88
55	134.35	80.49	6.8	124.41	-
65	173.96	82.47	7.0	-	80.83
75	153.40	-	7.2	197.69	-
			8.5	202.62	77.56
			9.0	-	78.58
			9.2	136.52	-

[a]%Relative activities of the crosslinked enzymes (CCAs and CLLAs) obtained with respect to their pristine forms. The enzyme activity data of CCLAs was sourced from the Figures 5 and 6 of reference 23, after obtaining permission from M/s. Elsevier Ltd., and transformed to %RA.

Fig. 1

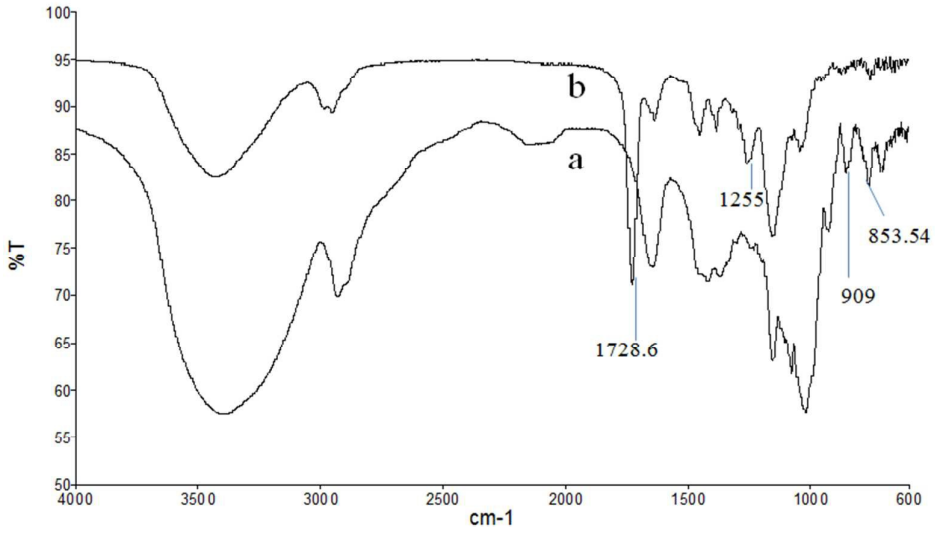


Fig. 2

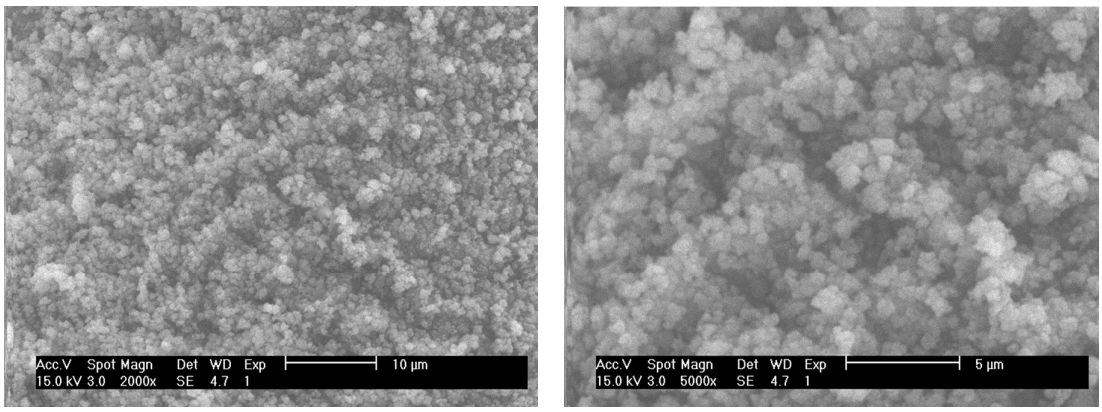


Fig. 3

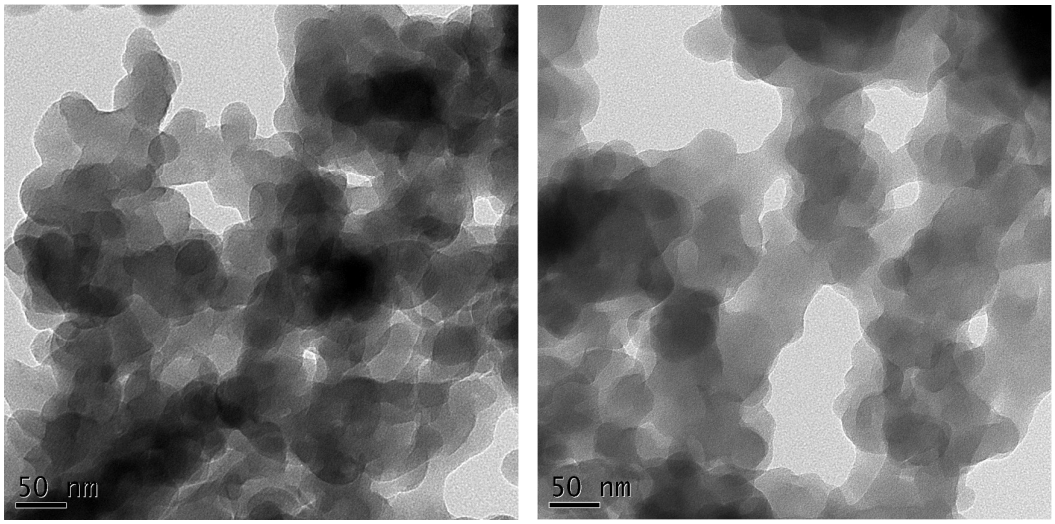
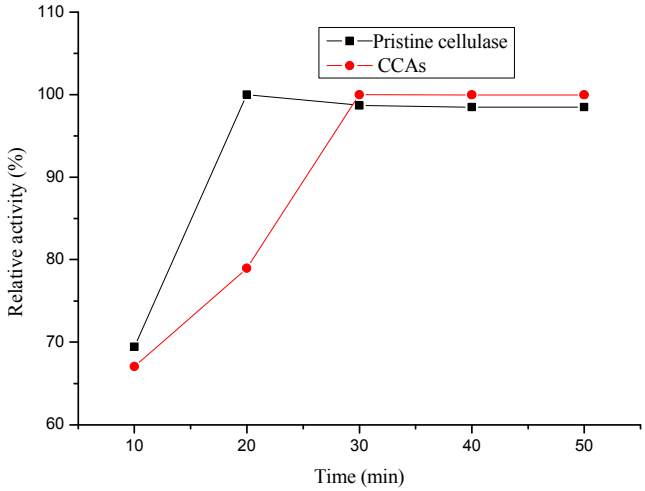


Fig. 4a



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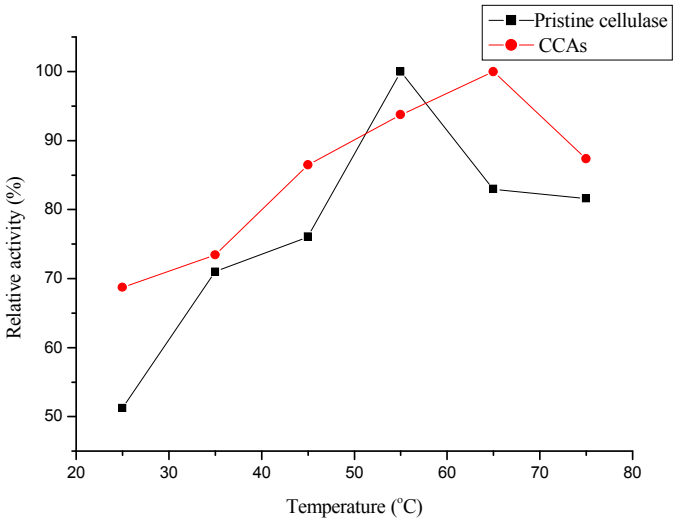
377 **Fig. 4b**



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379 **Fig. 4c**

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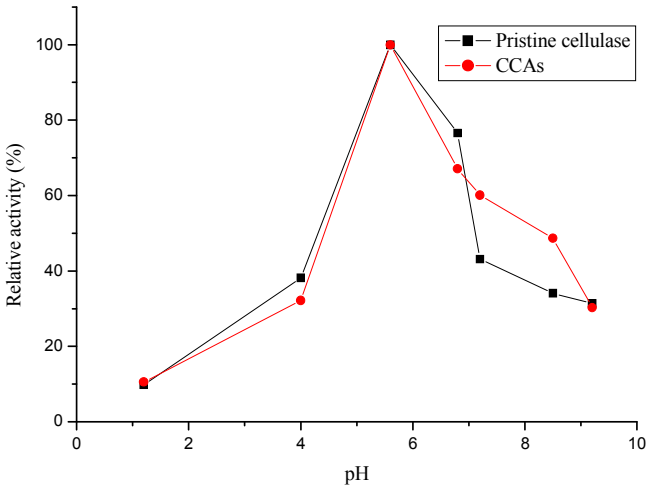

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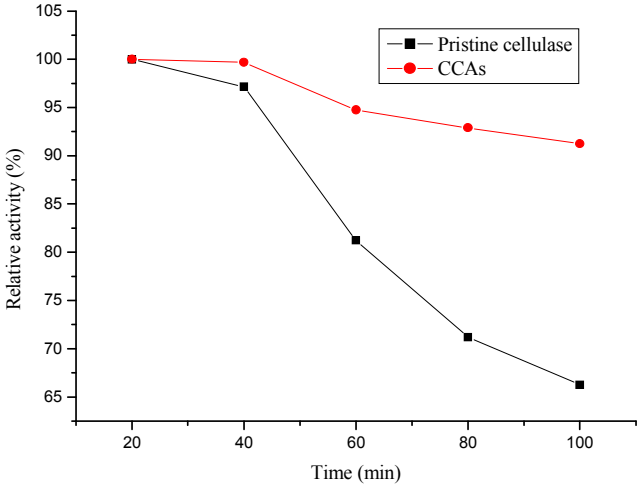
387 **Fig. 5**



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389 **Fig. 6**

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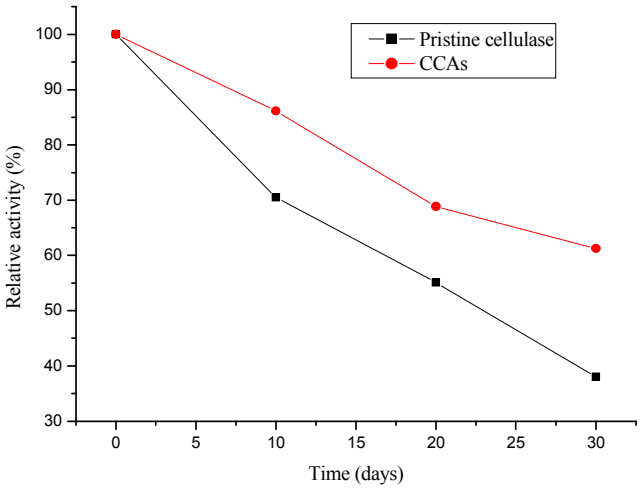

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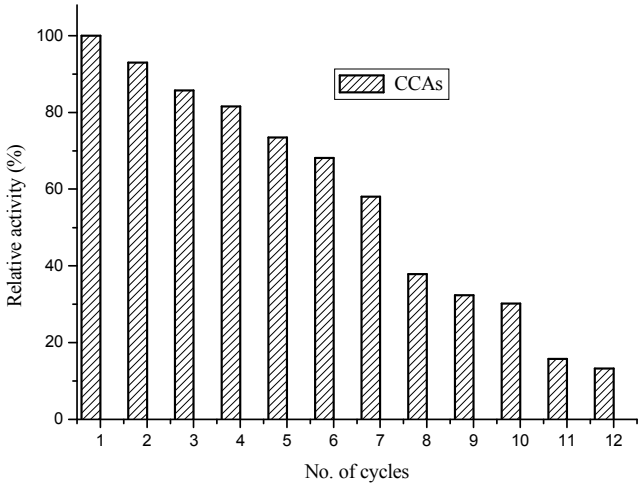
398 **Fig. 7**



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400 **Fig. 8**

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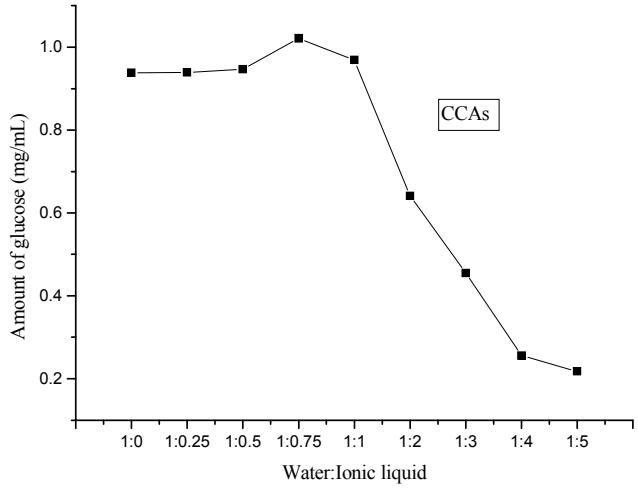

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Legends of Tables and Figures

Table 1: %Relative activity values for CCAs (EGDMA based crosslinking) and CLLAs[23] (*N,N* MBAAm based crosslinking)[a]

Fig. 1: FTIR spectra of pristine cellulase (a) and CCAs (b).

Fig. 2: SEM images of CCAs at magnification 2000X and 5000X (the CCAs are particle aggregates of spherical shape and as a whole have porous appearance).

Fig. 3: TEM images of CCAs (each enzyme particle is ~50 nm size).

Fig. 4a: Effect of time on the activities of pristine cellulase and CCAs (the measurements of enzyme activities were carried out in phosphate buffer (pH 5.5) at 50 ^{o}C. Relative activities were calculated by using the highest activity as 100 %).

Fig. 4b: Effect of temperature on the activities of pristine cellulase and CCAs (the measurements of enzyme activity were carried out in phosphate buffer (pH 5.5) at 20 and 30 min for pristine cellulase and CCAs respectively, at different temperatures. Relative activities were calculated by using the highest activity as 100 %).

Fig. 4c: Effect of pH on the activities of pristine cellulase and CCAs (measurements of enzyme activity were carried out at 55 ^{o}C and 65 ^{o}C for pristine cellulase and CCAs respectively at 20 and 30 min. Relative activities were calculated by using the highest activity as 100 %).

Fig. 5: Thermal stability of pristine cellulase and CCAs (enzymes were incubated at 60 ^{o}C and the measurements of enzyme activities were carried out in phosphate buffer (pH 5.6). Relative activities were calculated by using the highest activity as 100 %).

Fig. 6: Effect of storage time on activities of pristine cellulase and CCAs (pristine cellulase and CCAs were stored at 4 ^{o}C. The measurements of enzyme activities were carried out in phosphate buffer (pH 5.6). Activities were obtained upto 30 days taking optimum activity of both the pristine and the CCAs as 100%).

Fig. 7: % Relative activity plots for reusability upto twelve repeat cycles for CCAs (reactions were carried out in phosphate buffer (pH 5.6) at 65 ^{o}C. Relative activities were calculated by using the highest activity as 100 %).

Fig. 8: Activity of CCAs cellulase in different ratios of water and 1 butyl 3 methyl imidazolium acetate (measurements of enzyme activities were carried out at 30 min and 65 ^{o}C).