



Enzymatic hydrolysis of biomass with recyclable use of cellobiase enzyme immobilized in sol–gel routed mesoporous silica

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ABSTRACT

In this work, a new immobilized cellobiase enzyme system was used in the enzymatic hydrolysis of biomass for the generation of cellulosic ethanol. The application of the immobilized cellobiase enzyme enabled easy post-hydrolysis separation of the enzyme from the reaction mixture. The cellobiase enzyme was immobilized in porous solid silica host material via acid catalyzed non-surfactant templated sol–gel reaction of tetraethyl orthosilicate (TEOS). The pore size and porosity of the host material were controlled by varying the template (D-fructose) content. The immobilized cellobiase enzyme samples when used in the hydrolysis of pre-treated biomass yielded hydrolysis efficiency up to 74% (with FeCl₃ pre-treated biomass) and 81% (with oxalic acid pre-treated biomass) when compared to hydrolysis with free cellobiase enzyme. The solid immobilized enzyme samples could be easily recycled from the reaction mixture after the hydrolysis reaction. When re-used in multiple batches, the immobilized samples retained their enzymatic activity in biomass hydrolysis. The re-usability of the immobilized cellobiase with retained activity would actively bring down the enzyme cost. Thus, our immobilized cellobiase enzyme opens up great potential in the enzymatic hydrolysis of biomass for generating cellulosic ethanol, as it leads to overall process cost reduction.

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1. Introduction

For the last 15 years, a great amount of research was done to explore alternative energy sources [1,2]. In the production of ethanol as an alternative fuel, cellulosic biomass has great potential as an abundant renewable energy source [3,4]. In the last two decades, extensive research has been done on the conversion of lignocellulosic materials to ethanol [1,5–10], as it is the most abundant biomass available, which is capable of being converted to liquid fuel (ethanol) via enzymatic hydrolysis [11]. The method for converting lignocellulosic material to ethanol is mainly a two-step process [1]. The first step involves the hydrolysis of the cellulose in the lignocellulosic materials to fermentable reducing sugar (glucose). The second step in the process ferments the sugar to ethanol.

The hydrolysis step is usually done by enzymatic saccharification/hydrolysis of cellulose, catalyzed by cellulase enzymes, while the fermentation of glucose to ethanol is generally carried out by yeasts or bacteria [1]. The enzymatic hydrolysis of cellulose is affected by certain factors, which includes the crystallinity of cellulose fibers and the lignin and hemicellulose content [1,12]. The lignin and hemicellulose present in the biomass restricts the acces-

sibility of the cellulose to the cellulase enzymes, leading to reduced efficiency of the process. The removal of lignin and hemicellulose as well as the reduction of cellulose crystallinity are achieved by pretreatment of the biomass [1,12]. In this work, two different pre-treated biomasses were used; biomass pretreated with 2.5% FeCl₃ solution and biomass pretreated with 2.5% of 0.5 M oxalic acid solution. These two pretreatments, performed in our lab, have exhibited significantly higher hydrolytic efficiency with free cellobiase enzyme, when compared to biomass samples without any pretreatment.

During the enzymatic hydrolysis, cellulose is broken down to reducing sugars. This enzymatic hydrolysis is achieved by the application of highly specific cellulase enzymes [1]. The hydrolysis by the cellulase enzyme system primarily depends on three enzymes: β -1,4-endoglucanase, β -1,4-exoglucanase and cellobiase enzyme [13]. Cellulose is broken down to cellobiose units by the β -1,4-endoglucanase and β -1,4-exoglucanase, and then the cellobiase hydrolyzes the cellobiose units to produce glucose [1,3]. However, enzyme cost is one of the major hurdles in the path of large-scale commercialization of the enzymatic hydrolysis of cellulose [2,14], accounting for as much as 60% of the total process cost [15]. Enzyme recovery and recycling of enzymes is one of the most important and effective ways of increasing the efficiency of the enzymatic hydrolysis process by lowering the enzyme cost [14,16]. However, little research has been done on the application of recycled enzymes in

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the enzymatic hydrolysis of cellulose using pretreated wood as the primary feedstock [14].

In the present work, the primary objective was the application of a recyclable cellobiase enzyme system on the enzymatic hydrolysis of pre-treated biomass. Enzyme immobilization offers immense technological potential in this field as it extensively promotes enzyme reuse and overall process cost reduction [17–19]. The recyclable cellobiase enzyme system was developed through immobilization by direct encapsulation of the cellobiase enzyme in mesoporous silica, via a non-surfactant templated sol–gel route. The template and the enzyme were added to the precursor sol, and gelation was allowed to occur with the protein inside the silica matrix. Conventional sol–gel materials are microporous in nature, with diameter of the pore channels (that lead to the caged enzyme inside the matrix) typically ≤ 1.5 nm [20]. This smaller pore diameter restricted the diffusion of substrate molecules to the caged enzyme inside the host matrix, making the enzyme inside the matrix less accessible. The poor accessibility of the enzyme to the substrate in turn greatly hindered the catalytic activity of the immobilized enzyme [20]. In the mesoporous silica host material, the pore sizes are bigger to allow easy diffusion of the substrate to the enzyme caged inside the solid support material. D-fructose was used as the template or pore-forming agent, because it is very economical, biocompatible, and after synthesis, it could be easily removed from the silica pores by simply washing the samples in excess water. Also, unlike surfactant templated mesoporous material synthesis, the D-fructose templated route did not involve harsh and stringent reaction conditions, which are harmful to enzymes.

The immobilized cellobiase system enabled the easy post-hydrolysis recovery of the enzyme from the reaction media, leading to efficient re-use of the enzyme in multiple batches.

2. Experimental procedures

2.1. Materials

Tetraethyl orthosilicate (TEOS, 98%), D-(–)-fructose and sodium acetate trihydrate (99%) were obtained from Sigma–Aldrich. The cellobiase enzyme (from *Aspergillus niger*), liquid ≥ 250 U/g used was Novozym 188, a β -glucosidase obtained from Sigma–Aldrich. The cellulase from *Trichoderma reesei* ATCC 26921, lyophilized powder, ≥ 1 unit/mg solid was obtained from Sigma–Aldrich. The biomass used was poplar wood shavings obtained from Martin Millwork (Mercersburg, PA). The spectrophotometric assay reagents were included in a commercial glucose determination kit (Autokit Glucose, cat# 439-90901), obtained from Wako Pure Chemicals. All the reagents and materials were used as received without any further purification.

2.2. Synthesis of immobilized cellobiase samples

The immobilized cellobiase samples were prepared via acid catalyzed sol–gel reaction of TEOS with water. During the synthesis, D-fructose was used as the template or pore-forming agent. In a typical preparation of 52.19 g of 70% template content immobilized cellobiase sample, 9.0 g (0.5 moles) water and 0.63 g of 2 M HCl were mixed in a 500 ml 2 necked round bottomed flask under mild stirring at room temperature. The round-bottomed flask was fitted with a thermometer and a reflux condenser. After 5 min of stirring, 52 g (0.25 moles) TEOS was slowly added with stirring at room temperature. After the TEOS addition, the mixture was stirred at high speed under N_2 . The solution turned cloudy within 2 min of stirring, and then cleared out within another 5 min of stirring, with the reaction temperature rising to 35 °C. With further stirring, the reaction mixture turned cloudy again, and then cleared out within

a minute, with the temperature rising to 60 °C. The homogenous reaction mixture was refluxed at 60 °C for 1 h and then allowed to cool down to room temperature under constant N_2 purge.

After cooling down, the N_2 purge was removed, and the reaction mixture was connected to a high vacuum system, to remove the ethanol produced as a byproduct in the sol–gel reaction. The mixture was subject to high vacuum until it showed 50% weight loss. To this mixture, 70 g of 50% (by weight) of D-fructose in water solution was added under heavy stirring. The mixture was stirred and degassed simultaneously until a clear solution (solution A) was obtained. The solution A was then equally divided (by weight) into three 100 ml beakers. Each beaker contained 5 g of silica and 11.66 g of D-fructose. For each batch, an enzyme solution was prepared by adding 625 μ l of cellobiase enzyme in 5.0 ml of 50 mM sodium acetate buffer (pH 5.0). This enzyme solution was then added to the solution A under stirring at room temperature for 2 min. The reaction beaker was then sealed with parafilm and the reaction mixture was allowed to gel at 5 °C. After gelling, the 15–20 holes were made in the parafilm and the sample was stored at 5 °C for 48 h for solvent evaporation. After the solvent evaporation, the samples were connected to a high vacuum system at 0 °C until no further weight loss was observed.

After the drying process, the samples were crushed to 40-mesh size powder and stored at 5 °C. Immobilized cellobiase samples with 50% and 70% fructose content (with respect to silica) were prepared, to get mesoporous structured silica as the host material.

2.3. Characterization of immobilized cellobiase samples

The template (D-fructose) content in the immobilized cellobiase samples was determined from weight loss in air using Thermogravimetric Analysis (TGA) on a TA Q50 Thermogravimetric Analyzer (TA Instruments Inc., New castle, DE). Prior to analysis, the samples were heated to 80 °C in N_2 and kept isothermal at 80 °C in N_2 for 45 min. For the weight loss analysis, the samples were heated at 10 °C/min to 800 °C in air. The pore size, pore volume and surface area of the cellobiase-encapsulated silica powders were determined from nitrogen adsorption–desorption, done on a Quadrasorb SI Automated Surface Area and Pore size Analyzer (Quantachrome Instruments). The pore size and surface area of the samples were calculated using the Quantachrome QuadraWin software (Version 5.02).

2.4. Procedure for enzymatic hydrolysis of biomass

For our biomass hydrolysis, we have used two enzymes: cellulase enzyme from *Trichoderma reesei*, and cellobiase enzyme from *Aspergillus niger*. The cellulase from *Trichoderma reesei* was used to break the cellulose (in biomass) into cellobiose units. The cellobiose units were further broken down to glucose units by cellobiase enzyme [1,13]. Instead of free cellobiase enzyme, we have used immobilized cellobiase enzyme in our biomass hydrolysis, along with free cellulase (from *Trichoderma reesei*). The objective of using immobilized cellobiase was to enable reusability of the enzyme in multiple reaction batches. Since cellulose is water insoluble substrate, free cellulase had to be used, so that the enzyme can travel to the cellulose substrate. However, the cellobiose units generated from the hydrolysis are water soluble, and hence can travel to the enzyme [2]. Hence, we have used immobilized cellobiase enzyme.

For the enzymatic hydrolysis of biomass, cellulase enzyme from *Trichoderma reesei* was first prepared. In this preparation, 5 mg of the lyophilized powder was taken in a 5 ml volumetric flask, and 5 ml de-ionized water was added to it. The mixture was shaken until the powder completely dissolved in the water. A sodium acetate buffer solution was prepared according to the Sigma–Aldrich cellulase assay procedure. In this method, a 50 mM solution of sodium

acetate trihydrate (99%) in distilled water was prepared. The pH of the solution was adjusted to 5.0 using 1 M hydrochloric acid (HCl). Prior to hydrolysis experiments, the immobilized cellobiase in the silica samples were washed in the sodium acetate buffer to remove the D-fructose, and make the enzyme inside the pores accessible by substrates. For washing, appropriate amount of the immobilized samples were taken in 15 ml falcon tubes, such that the amount of cellobiase enzyme in each tube was 25 μ l. To these tubes, 10 ml of the pH 5 buffer solutions was added, the tubes were sealed and agitated on a rocker at room temperature for 15 h. After washing, the liquid was decanted out.

As discussed in Section 1, pre-treated biomass was used for this study. To the washed cellobiase samples, appropriate amounts of pre-treated biomass and fresh buffer solution were added. The falcon tubes contained 25, 50, 100, 200 and 400 mg/ml of pre-treated biomass. After this, 0.5 ml of the cellulase enzyme solution was added to each tube. The tubes were then sealed and shaken on a vortex mixer for 10 s and then agitated at 37 °C for 2 h in a water bath.

After 2 h of hydrolysis, the tubes were removed from the water bath and placed on ice until the contents were cooled. After cooling, the tubes were centrifuged at 500 rpm for 2 min. The supernatant was decanted into a sample vial and the glucose content in it was determined by the procedure as given in Wako Glucose Autokit (Wako Pure Chemicals), expressed as milligrams of glucose per milliliter of the solution (mg/ml). The activity of the immobilized samples was expressed in terms of milligrams of glucose produced per milliliter of the solution (mg/ml).

3. Results and discussions

3.1. Thermogravimetric analysis (TGA)

The template (fructose) content in the as synthesized immobilized cellobiase samples were analyzed by the weight loss obtained in the thermogravimetric analysis (TGA). The samples were heated in air at 10 °C/min–800 °C, and Fig. 1 shows the TGA thermographs of the as synthesized samples. The weight losses obtained are listed as TGA weight loss in Table 1. The observed weight losses in the samples are due to the degradation of the fructose present in the samples. From the data listed in Table 1, we can confirm that the fructose content in the samples, as calculated from the reaction stoichiometry, are proportional to the TGA weight losses obtained. The weight loss (<10%) observed in 0%F sample can be attributed to the presence of unreacted ethoxy groups, due to incomplete sol–gel reaction of TEOS [21]. A weight loss (<10%) in samples without any template content has been reported earlier [20,22].

3.2. Determination of textural properties of the silica host material

The Brauner–Emmett–Teller (BET) surface area, pore size and the pore volume of the silica host materials were determined from the nitrogen adsorption–desorption isotherms and are listed in Table 1. Prior to analysis, the template was extracted from the samples by extensively washing the powder samples in a large excess

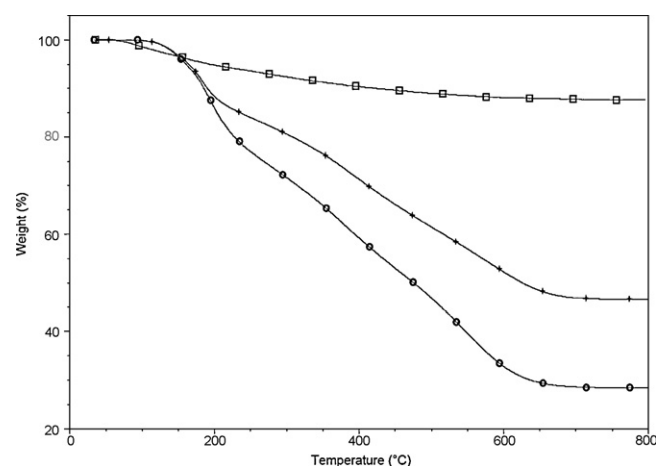


Fig. 1. TGA thermographs of the as-synthesized cellobiase encapsulated silica samples for verifying the added template (fructose) content. The nomenclatures of the samples are described in Table 1. Immobilized sample with 0% fructose content (0%F) is represented by (□); immobilized sample with 50% fructose content (50%F) is represented (+); immobilized sample with 70% fructose content (70%F) is represented by (○).

of distilled water. The nitrogen adsorption–desorption analysis was done at –196 °C, and Fig. 2 shows the adsorption–desorption isotherms. The 70%F and 50%F samples exhibit type IV isotherm with H2 hysteresis loop, which are characteristic of molecular sieves having pore structures in the mesoporous range [23–25]. The 0%F sample exhibited a reversible type I isotherm, characteristic of microporous structure [23,26].

Non-local density functional theory (NLDFT) method was used to calculate the pore size distribution and the pore volumes while the BET equation was used to determine the surface area of the silica host materials [27]. From the pore size distribution (Fig. 3) we can see that 70%F and 50%F samples exhibit peak maxima in mesoporous range (3–3.5 nm). The 0%F sample showed peak maxima in microporous range (<2 nm). The observed pore volumes in the 50%F and 70%F samples (0.52 and 0.57 cm³/g respectively) as listed in Table 1, are also larger than the conventional microporous sol–gel materials (≤ 0.25 cm³/g), and lie in mesoporous range [20]. Hence, from the textural properties of the silica host material, it is evident that in 70%F and 50%F samples, the cellobiase enzyme is immobilized in mesoporous silica host material.

3.3. Enzymatic activity of immobilized cellobiase in the hydrolysis of biomass

The enzymatic hydrolysis of biomass with the immobilized cellobiase enzyme was done by the procedure as described in Section 2.4. As discussed earlier, pre-treatment of the biomass is essential for enzymatic hydrolysis. In this work, two different pre-treated biomasses, 2.5% FeCl₃ treated biomass and 2.5% (w/v) of 0.5 M oxalic acid treated biomass were used.

For the FeCl₃ pre-treated biomass and oxalic acid pre-treated biomass, the enzymatic activities of immobilized cellobiase samples with varying biomass concentrations were determined. The

Table 1

Template content, TGA weight loss and textural properties of the immobilized cellobiase samples.

Sample	Weight % template added ^a	TGA weight loss (%)	BET surface area (m ² /g)	Pore diameter (nm) ^b	Pore volume (cm ³ /g)
0%F	0	<10	280	1.5	0.15
50%F	50	51	780	3.1	0.52
70%F	70	71	796	3.4	0.57

^a Weight % template added was calculated from the reaction stoichiometry, assuming complete conversion of TEOS to silica.

^b Obtained from the peak maxima of the pore size distribution plot.

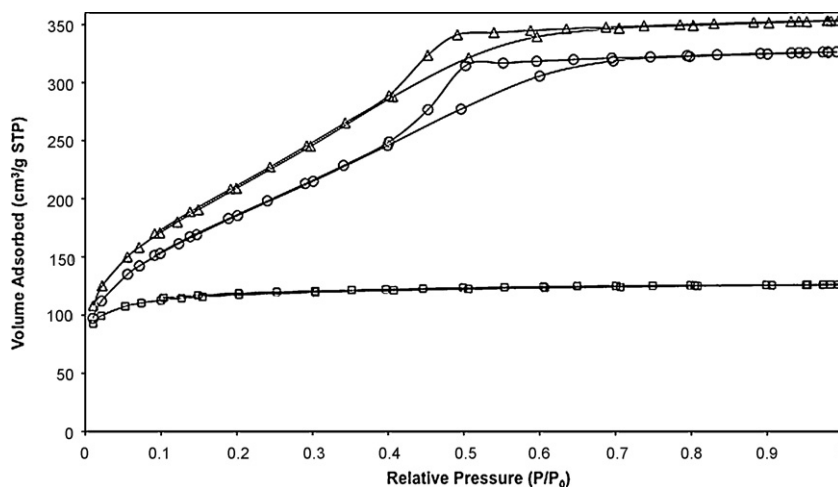


Fig. 2. Nitrogen adsorption–desorption isotherms of the silica host materials of the immobilized samples. The template was extracted out of the samples by washing with excess water. Sample with 0% fructose content (0%F) is represented by (\square); sample with 50% fructose content (50%F) is represented by (Δ); sample with 70% fructose content (70%F) is represented by (\circ).

Table 2
Maximum activity, hydrolysis efficiency and V_{\max} values of immobilized cellobiase and free cellobiase enzyme in biomass hydrolysis. 2.5% FeCl_3 pre-treated biomass and 2.5% 0.5 M oxalic acid pre-treated biomasses were used for the hydrolysis.

Sample	FeCl_3 pre-treated biomass			Oxalic acid pre-treated biomass		
	Max activity (mg/ml)	Hydrolysis efficiency (%)	V_{\max} (mg/ml hr)	Max activity (mg/ml)	Hydrolysis efficiency (%)	V_{\max} (mg/ml hr)
0%F	0.58 ± 0.06	54	0.31	0.59 ± 0.12	50	0.31
50%F	0.79 ± 0.03	73	0.42	0.84 ± 0.06	72	0.54
70%F	0.80 ± 0.12	74	0.48	0.95 ± 0.16	81	0.44
Free cellobiase	1.08 ± 0.14	100	0.74	1.18 ± 0.14	100	0.65

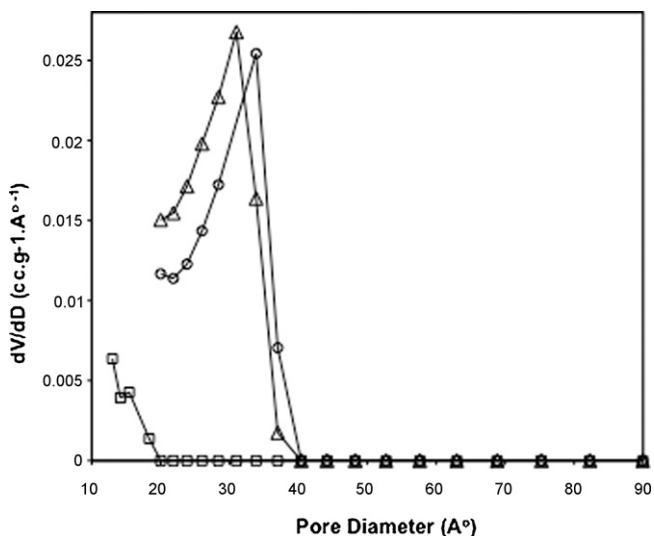


Fig. 3. Pore size distribution of the host silica materials of the immobilized samples. Sample with 0% fructose content (0%F) is represented by (\square); sample with 50% fructose content (50%F) is represented by (Δ); sample with 70% fructose content (70%F) is represented by (\circ).

maximum activities of the immobilized cellobiase samples as well as the native free cellobiase enzyme obtained are listed as max activity (mg/ml) in Table 2. The efficiency of our immobilized system compared to the free enzyme is listed as hydrolysis efficiency (%) in Table 2, and was calculated according to Eq. (1):

$$\text{Hydrolysis Efficiency (\%)} = \frac{\text{Max activity of sample}}{\text{Max activity of free cellobiase}} \times 100 \quad (1)$$

From the enzymatic activities listed in Table 2, it is demonstrated that the immobilized cellobiase system attained up to 74% (70%F for FeCl_3 pre-treated biomass) and 81% (70%F for oxalic acid pre-treated biomass) hydrolysis efficiency, in the biomass hydrolysis. The hydrolysis efficiency of 0%F samples are lower, at 54% for FeCl_3 pre-treated biomass and 50% for oxalic acid pre-treated biomass. This observed difference in max activity and hydrolysis efficiency could be attributed to the silica host microstructure. As listed in Table 1, the samples with higher fructose contents (70%F and 50%F) are mesoporous and have bigger pore diameters (3.4 nm and 3.1 nm respectively) compared to 0%F samples, which is microporous in nature (pore diameter <2 nm). The bigger pore diameters allowed easier diffusion of the substrate (cellobiose) through the pores, thus increasing the accessibility of the enzyme. In the 0%F sample, the smaller pore diameter hindered the diffusion of the substrate, thereby restricting the accessibility of the enzyme. As evident from Table 1, the surface area and the pore volume of the 70%F and 50%F samples also are higher compared to the 0%F sample. The high surface area signifies greater concentration of pores in the host material. High template (fructose) content opened up more interconnected pores/channels in the silica, thereby increasing the porosity of the host silica. Hence, the bigger pores and higher porosity in 70%F and 50%F samples led to higher enzymatic activities, compared to the 0%F sample.

It must be noted that the cellobiase enzyme molecules are trapped within the silica matrix, via direct encapsulation by gelation of the silica precursor sol around the protein molecules. The template actually increased the diameter and concentration of the interconnected open pore channels, leading to the caged enzyme inside the silica matrix. In 70%F and 50%F samples, there is template-induced porosity, which makes the pore sizes bigger, thus promoting easy diffusion of substrate through the pores, to the enzyme caged within the host matrix. In 0%F sample, the pore

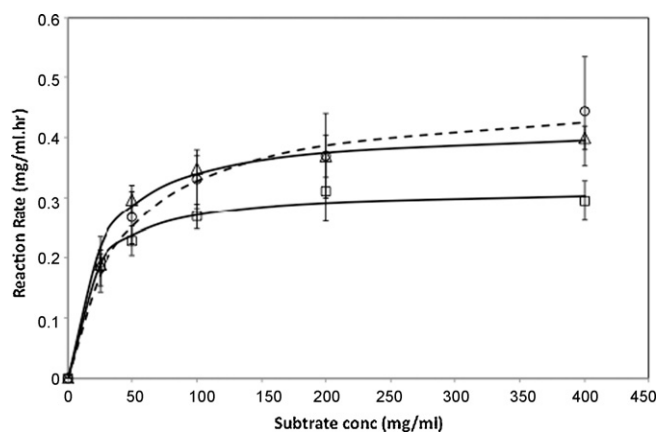


Fig. 4. Michaelis–Menten plots for the immobilized samples with FeCl_3 pre-treated biomass. The x-axis represents the biomass concentration. The y-axis represents reaction rate (v_0). Immobilized cellobiase sample with 0% fructose content (0%F) is represented by (\square); immobilized cellobiase sample with 50% fructose content (50%F) is represented by (Δ); immobilized cellobiase sample with 70% fructose content (70%F) is represented by (\circ).

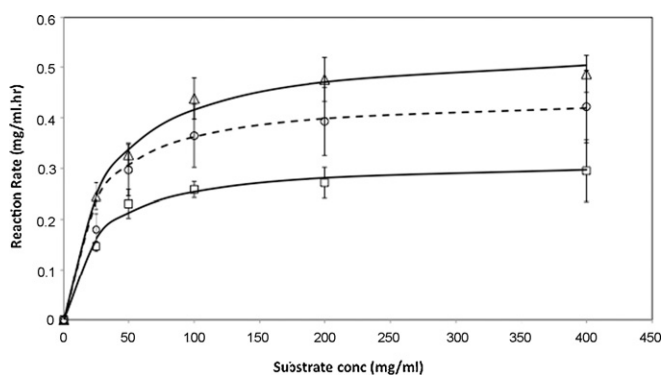


Fig. 5. Michaelis–Menten plots for the immobilized samples with oxalic acid pre-treated biomass. The x-axis represents the biomass concentration. The y-axis represents reaction rate (v_0). Immobilized cellobiase sample with 0% fructose content (0%F) is represented by (\square); immobilized cellobiase sample with 50% fructose content (50%F) is represented by (Δ); immobilized cellobiase sample with 70% fructose content (70%F) is represented by (\circ).

diameters are smaller (due to absence of template-induced porosity), thus reducing accessibility of the enzyme caged within the silica microstructure, by the substrate.

The Michaelis–Menten kinetics for the immobilized cellobiase samples in the hydrolysis of biomass was studied according to Eq. (2) [28]:

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (2)$$

where v_0 is the rate of enzyme activity or the reaction rate of hydrolysis at any given substrate (biomass) concentration, $[S]$ is the substrate concentration, V_{\max} is the maximum rate of conversion or the maximum rate of hydrolysis and K_M is the substrate concentration at which the rate of reaction or rate of hydrolysis is half of V_{\max} . The Michaelis–Menten plots for the immobilized cellobiase samples are shown in Figs. 4 and 5, and the V_{\max} values obtained for the immobilized cellobiase samples as well as the free cellobiase samples for the two pre-treated biomass hydrolysis are listed in Table 2. From the data listed in Table 2, it is demonstrated that our immobilized cellobiase samples yielded V_{\max} values $\sim 65\%$ (70%F with FeCl_3 pre-treated biomass) and 83% (50%F with oxalic acid pre-treated biomass) when compared to biomass hydrolysis with free cellobiase enzyme. The 0%F showed a relatively lower V_{\max} value. This observation is in accordance with the max activity

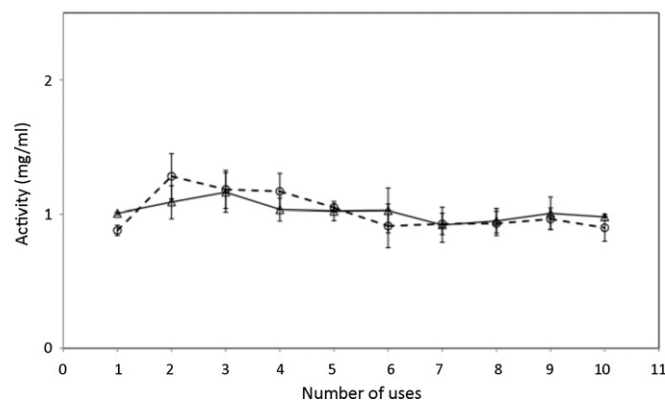


Fig. 6. Activity of 70%F and 50%F immobilized cellobiase samples, recycled and used in multiple batches. The biomass used was pretreated with 2.5% (w/v) 0.5 M oxalic acid solution. All the runs were done in pH 5 sodium acetate buffer, at 37°C . Immobilized cellobiase sample with 50% fructose content (50%F) is represented by (Δ); immobilized cellobiase sample with 70% fructose content (70%F) is represented by (\circ).

values. The smaller pore size and lower porosity greatly restricted the accessibility of the enzyme inside the caged structure. This led to lower rates of reaction for the 0%F sample, as evident from the low V_{\max} value obtained.

Hence, from the hydrolysis efficiency results reported, it is evident that our immobilization method produced highly efficient immobilized enzyme systems, retaining up to 80% hydrolysis efficiency after synthesis.

3.4. Recyclable use of immobilized cellobiase in biomass hydrolysis

As discussed earlier, the most important aspect and the primary goal of this work was to generate an immobilized enzyme system, which could be easily separated from the biomass reaction mixture, and readily be recycled for multiple uses. To enable multiple use of our immobilized enzyme system in biomass hydrolysis, we designed a two-step hydrolysis process, based on the understanding that during the enzymatic hydrolysis, the cellulase enzyme first breaks down the cellulose into cellobiase units, and thereafter, the cellobiase units are broken into glucose by the cellobiase enzyme [1,3]. The biomass used for the recyclable application was FeCl_3 pre-treated biomass and oxalic acid pre-treated biomass. The 50%F and 70%F immobilized cellobiase samples were used at the biomass concentration of 400 mg/ml. In the first step of our experimental design, the biomass (cellulose) was broken down to cellobiase by cellulase enzyme. For this, 400 mg biomass was taken in a 15 ml falcon tube. To this tube, 0.5 ml of cellulase enzyme solution and 2 ml sodium acetate buffer was added; the tube was sealed and mixed in a vortex mixer for 10 s. Then, the tube was agitated at 37°C for 2 h. After the hydrolysis of cellulose to cellobiase, we go the second step, where, the liquid phase of the reaction mixture containing the cellobiase was taken out and added to another 15 ml falcon tube which contained appropriate amount of either the 50F or the 70F immobilized cellobiase samples (enzyme content was $25 \mu\text{l}$). It was then mixed in a vortex for 10 s, and then agitated at 37°C for 2 h. After the hydrolysis of the cellobiase, the glucose content in the liquid phase was determined by the Wako Glucose Autokit method, as described in Section 2.4. The activity of the immobilized cellobiase was expressed in terms of milligrams of glucose per milliliter of solution. After the run, the immobilized samples were filtered out, and the whole process was repeated for the next subsequent run. In this way, the immobilized cellobiase samples were reused in 10 multiple batches.

Fig. 6 shows the activity of the immobilized cellobiase with oxalic acid pre-treated biomass hydrolysis in multiple uses. From the results, it is clearly demonstrated that with up to 10 reuses, our immobilized cellobiase samples maintained their activities during the biomass hydrolysis. This retained activity also confirms that the enzyme is well encapsulated within the silica matrix with negligible leaching. Cellobiase is a globular enzyme with a diameter of 51 Å [2], and hence bigger than the diameter of the pore channels. The smaller diameter of the pore channels in the silica host material thus prevented the enzyme from coming out of the caged structure. The elution of the enzyme from the host material was also checked by washing the immobilized samples in pH 5 sodium acetate buffer and assaying the washing solution. All the washing solutions showed negligible enzymatic activity.

The Recycled cellobiase used with FeCl₃ pre-treated biomass also exhibited similar results. Hence, it is clearly established that with our experimental design, recyclable application of our immobilized cellobiase system is attainable in the enzymatic hydrolysis of biomass with high hydrolysis efficiency.

4. Conclusion

In this work, the immobilized cellobiase enzyme system was used in the enzymatic hydrolysis of biomass. The cellobiase enzyme was immobilized in mesoporous silica host material via the sol–gel reaction of TEOS with water and D-fructose was used as a template or pore-forming agent. The immobilized cellobiase system, when applied in the hydrolysis of pre-treated biomasses, yielded hydrolysis efficiency up to 74% and 81%, when compared to hydrolysis with free cellobiase enzyme. A new experimental design was set up to allow easy post-hydrolysis separation of the immobilized enzyme from the reaction mixture. The recycled enzyme, when used in multiple batches, maintained their activities in the biomass hydrolysis. Thus, we have demonstrated that our immobilized cellobiase system, when used in the enzymatic hydrolysis of biomass enabled the recyclable use of the enzyme. This recyclability enabled the re-use of the enzyme maintaining the high hydrolysis efficiency in biomass hydrolysis.

Our immobilized cellobiase enzyme thus opens up great potential in the production of ethanol as biofuel from the hydrolysis of renewable sources such as biomass. The recycling and reusability of the immobilized enzyme system would actively bring down the enzyme cost, leading to an overall decrease in the cost of production of the biomass hydrolysis process.

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