RSC Advances

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2013, **3**, 23604

CelB and β-glucosidase immobilization for carboxymethyl cellulose hydrolysis

Clara T. H. Tran,* Neil J. Nosworthy, Alexey Kondyurin, David R. McKenzie and Marcela M. M. Bilek

Received 15th July 2013
Accepted 1st October 2013

DOI: 10.1039/c3ra43666g

www.rsc.org/advances

Cellulose hydrolysis is an important step in the production of bioethanol from cellulosic biomass. Two key cellulase enzymes, celB and β-glucosidase, were covalently immobilised on polystyrene treated with plasma immersion ion implantation (PIII) which creates radicals that form covalent bonds. The immobilized enzymes were used to produce glucose from carboxymethyl cellulose (CMC), a solubilised form of cellulose. CelB from *Caldicellulosiruptor saccharolyticus* is a thermophilic cellulase with both endoglucanase and exoglucanase activity. The highest activity of the immobilised celB on PIII treated surfaces was achieved when their immobilisation is carried out at a pH in the range 5–6.5. The immobilized celB on the PIII treated surface had the same activation energy as free celB showing substrate accessibility is not affected by the presence of the surface. The V_{max} and K_m values of immobilized celB were found to be comparable to those of equal free celB concentrations. The areal density of immobilized celB on the PIII treated surface was estimated to be 0.3 μg cm^{-2}. The polystyrene surface with immobilized celB at 45 °C could be reused over four times (23 hours each) with approximately 30% total activity loss. High ratios of β-glucosidase to celB were found to enhance the activity of immobilized celB for CMC hydrolysis.

1. Introduction

The production of ethanol from agricultural sources is a fast growing industry with approximately 400% growth over the last decade. The development of new enzymes is opening the way to the so called "second generation" technology where ethanol is derived from low cost cellulosic biomass sources such as municipal waste, agricultural waste and forest waste. At the moment, cellulosic ethanol production is limited to pilot scale plants due to the relatively low efficiency of hydrolysing the biomass into fermentable sugars. The hydrolysis process comprises many transformations in which cellulose and hemicellulose are hydrolysed into shorter fragments and finally into the short chain sugars including hexoses and pentoses. This process is usually implemented with mixtures of enzymes known as cellulases. There are several commercial cellulolytic enzymes for cellulosic biomass hydrolysis in the market such as the "Cellic" series from Novozyme and the "Accelerase" series from Genencor. A dosage of 50 kg of Cellic Ctec3 is required per 1000 kg of ethanol while at least 250 kg of a competing enzyme is needed to create the same amount of ethanol.[1] "Accelerase Trio" from Genencor requires a dosage of 30–160 l per ton of biomass.[2] Although it is difficult to make a comparison due to differing units, it is clear that large quantities of enzymes are currently required for industrial scale processes. Therefore the enzyme cost to hydrolyse the low value and abundant cellulosic biomass at these levels is high, reducing the economic benefits of cellulosic ethanol. One possible way of reducing the effective cost of these enzymes would be to re-use the enzymes.

Cellulases are enzyme mixtures which hydrolyse the β-1,4 glucosidic linkages in cellulose. Cellulases consist of three main groups of enzymes: endoglucanases (these randomly cleave internal bonds to create new chain ends), exoglucanases (these cleave every two or four units from the ends to create cellobiose or cellotetraose) and β-glucosidase (this cleaves cellobiose into two glucose units). Beta-glucosidase, the last enzyme in the hydrolysis process, is required in large quantities. A new thermophilic β-glucosidase has recently been immobilized on a plasma immersion ion implantation (PIII) treated polystyrene surface and has been successfully reused, substantially increasing its economic efficiency.[3]

PIII treatment is a method in which energetic ions bombard the polymer surface, breaking bonds in polymer chains and creating radicals in the surface and subsurface regions.[4] The surface exposed to air after PIII treatment becomes more hydrophilic with the appearance oxygen containing groups.[5] The radicals on the PIII treated polystyrene surface can be detected using electron paramagnetic resonance.[6] Cells, proteins and enzymes have been reported to be covalently attached to the PIII treated surfaces within a short incubation

Applied and Plasma Physics, School of Physics (A28), University of Sydney, Sydney, New South Wales 2006, Australia. E-mail: C.Tran@physics.usyd.edu.au; Tel: +61 2 91141338

time.[3,7–10] Unlike other covalent immobilization using chemical linker groups, these biomolecules bind to the PIII treated surface without requiring any chemical linkers. Experiments with copolymers containing carboxyl groups[11] showed that carboxyl groups appearing upon oxidation of the surface are not responsible for the covalent bonding of the biomolecules. A direct link between the surface embedded radicals and the covalent attachment was established[4] by the correspondence of the time dependences of these processes.

Covalent binding is known to be stronger than physical adsorption and ionic binding and can prevent enzyme desorption from the carrier.[12] Because the covalent bonds are irreversible, it is important that the enzyme keep its activity while being immobilized. Most polymers have hydrophobic surfaces. The adsorption of enzymes on a polymer surface can induce a change of enzyme conformation due to the spreading of the hydrophobic domains of the enzyme molecule on that surface. As a consequence, its activity decreases. In contrast, the polymer surface after the PIII treatment is more hydrophilic.[5,8,11] When an enzyme molecule approaches the PIII treated surface, less change in its conformation is required. Hence, its catalytic activity is retained. β-Glucosidase[3] and horseradish peroxide[8] attached on the PIII treated polymers have been observed to have higher activity than on the untreated surfaces.

In this paper, celB, a thermophilic enzyme performing the functions of both endoglucanases and exoglucanases,[13] was immobilised and demonstrated its use together with immobilised thermophilic β-glucosidase in an enzymatic hydrolysis process. The use of only two enzymes is a simplification of the process that may lead ultimately to cost advantages. In order to obtain the best performance, thermophilic enzymes were used to allow the operation at higher temperatures while preserving enzyme function.

It has been reported in two papers that celB consists of three domains, Saul *et al.*[13] label the domains A, B and C while Park *et al.*[14] label them GH10, CBM3 and GH5. The middle domain (B or CBM3) is a binding domain which helps celB to bind to an insoluble cellulose substrate. When used with the solubilised cellulose, carboxymethyl cellulose (CMC), domain C was found to have CMCase activity (activity to hydrolyse CMC) while domain A was found to have exoglucanase activity. HPLC analysis showed that cellobiose is the main product of celB when using cellooligosaccharide[15] or "Avicel" (crystalline cellulose)[14] as substrate. Cellobiose inhibits celB catalytic activity when present in high concentrations.[14] In the presence of β-glucosidase, cellobiose is hydrolysed into glucose quickly, hence reducing the inhibition effect.

The aims of this work are the identification of the optimum parameters for the immobilization of recombinant celB onto PIII treated polystyrene (PS) surfaces, determining the effects of immobilisation on the thermal stability and activity; and assessing the reusability of immobilized celB and its synergy when used with surface immobilised β-glucosidase. This work has important implications for the production of ethanol using immobilised enzymes. The possibility of a fully immobilised enzyme scheme would allow the maximum benefits of immobilisation to be realised.

2. Materials and methods

2.1 Expression and purification of recombinant celB from *Caldicellulosiruptor saccharolyticus*

Genomic DNA from *Caldicellulosiruptor saccharolyticus* was a gift from Serve Kengen. The PCR product for the protein (UniProtKB/Swiss-Prot Accession number P10474) was sub-cloned into pET 3C (Novagen) using restriction enzymes NdeI and BamH1, transformed into *E. coli* BL21 (DE3) Star (Invitrogen) and the product was verified by DNA sequencing. The protein was expressed with and without the putative signal peptide. For this paper, the expressed protein was used without the putative signal peptide according to the UniProtKB/Swiss-Prot data base (residues 29–1039).

E. coli cells were grown in culture, harvested and resuspended in 100 ml of 10 mM Tris, 100 μM EDTA, 100 μM EDTA pH 8 and lysed using a Rannie homogenizer. After lysis the supernatant was heated to 70 °C for 30 minutes and centrifuged. The supernatant was subsequently applied to a Q Sepharose ion exchange column and proteins were eluted using a 0–0.5 M NaCl gradient. The peak containing celB was pooled and precipitated with 50% ammonium sulphate overnight at 4 °C. The precipitation was dissolved in 10 mM Tris at pH 8 and then applied to a Sephacryl S-200HR gel filtration column and run using 25 mM NaCl, 10 mM Tris buffer at pH 8. The peak containing celB was pooled and applied to a Mono Q column again. The column was washed with 10 mM phosphate buffer pH 8 and eluted with a 0–0.5 M gradient of NaCl in 10 mM phosphate pH 8. Fractions containing celB were pooled and dialysed against 10 mM phosphate buffer pH 7, aliquoted and stored frozen at −80 °C. Protein concentration was determined using the molar extinction coefficient ($\varepsilon_{280} = 298\ 565\ M^{-1}\ cm^{-1}$). The yield of celB was approximately 4 mg l^{-1}.

2.2. PIII treatment of polystyrene and celB immobilization

Polystyrene sheet (0.19 mm thick from Goodfellow) was attached to a conductive sample holder connected to an electrode with a pulsed bias applied to it. A stainless steel mesh was placed approximately 50 mm from the sample surface to reduce charging effects.[16] The sample holder was immersed in nitrogen plasma (generated by RF power, 100 W, 13.56 MHz) and periodically bombarded by nitrogen ions under a bias of −20 kV, 50 Hz for 20 μs. The ion fluence over the treatment was 5×10^{15} ions per cm^2. Samples were treated on both sides and used within 24 hours after treatment.

Samples (PIII treated and untreated PS) were incubated in celB solution on a rocker overnight at room temperature (approximately 22 °C). All celB solution for incubation was prepared with a concentration of 95 μg ml^{-1} using acetate buffer (10 mM, pH 5.5) unless stated. After immobilization, samples were transferred to a new container and washed four times with the same acetate buffer to remove any unbound molecules.

2.3 Test of covalent binding

This experiment was performed to illustrate the different mechanism of celB attachment onto PIII treated surfaces

compared to untreated surfaces. CelB was immobilized on PIII treated and untreated PS, washed using acetate buffer and milli-Q water and dried at room temperature before subjected to Fourier Transform Infrared Spectroscopy-Attenuated Total Reflection (FTIR-ATR) analysis. Spectra from the sample surfaces were recorded using a Digilab FTS 7000 FTIR spectrometer with germanium crystal ATR accessory (Harrick Inc., USA) and an incident angle of 45°. Five hundred scans were done on each analysis with a resolution of 4 cm^{-1}.

After that, both samples were treated with 2% Sodium Dodecyl Sulphate (SDS) at 70 °C for an hour. SDS washing is commonly used to test for covalent binding in the literature.[4,10,17] After the treatment, samples were washed three times with milli-Q water and dried in a desiccator before being analysed again with FTIR-ATR.

The peaks corresponding to the PS were removed from the protein-containing spectra by subtraction of the spectra of the same surfaces receiving identical exposure to buffer and atmosphere except without protein. Spectra after subtraction before and after SDS treatment were normalized to account for pressure differences between sample and ATR crystal during measurement using the intensity of the line of PS at 1492 cm^{-1} in the original spectrum with protein.

2.4 Influence of pH on carboxymethyl cellulose (CMC) hydrolysis using free celB and immobilized celB

Enzyme activity is affected by the pH of the buffer. In order to determine the optimum pH for celB attachment on PIII treated surface, the optimum pH of free celB to hydrolyse CMC in solution was studied. CMC solutions (6.7 mg ml^{-1}) were prepared with different buffers (pH = 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) at a concentration of 50 mM. Acetate was used for buffer with pH 4.5–5.5 and MES was used for buffers with pH 6.0–7.0. CelB was added to 15 ml of these CMC solutions at 0.76 μg ml^{-1} and incubated in a water bath at 55 °C. Samples were taken after 24 hours to analyse reducing sugar using DNS assay. Optimum pH of free celB is the pH which shows the highest activity.

The PIII treated surface has an increasing negative charge in buffers ranging from pH 4–7.[18] When celB comes to the PIII treated surface and attaches to the surface, the charge on celB molecules and the surface determine the enzyme's orientation and subsequently may or may not block the catalytic domain. Therefore, it is important to study the good range of pH for celB immobilization. Six different buffers (pH = 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0) were prepared with low concentration (10 mM). PIII treated surfaces (2 × 4 cm^2) were incubated with celB in different buffers overnight; washed four times with the same buffers and assayed in 10 ml CMC solution (6.7 mg ml^{-1}, pH 5.5, 50 mM acetate). The reducing sugars created in each pH buffers were determined using DNS assay to choose the optimum pH for celB attachment.

DNS assay: an aliquot of 100 μl sample was added to an Eppendorf tube with the same volume of 1% DNS (dinitrosalicylic acid) reagent.[19] The mixture was heated at 90 °C in a water bath for 10 minutes before adding Rochelle salt (33.3 μl) to stabilize the colour. Absorbance was done by spectrophotometer (Amersham, Pharmacia Ultrospec 3100 Pro Spectrophotometer) at 540 nm. CMC solution or milliQ water was added to DNS reagent in the same procedure and used as a blank. Cellobiose and glucose were used to build standard curves for absorbance conversion into relevant concentration.

2.5 Thermal stability of free celB in solution and immobilized celB on PIII treated surface

The purpose of this experiment is to investigate the changing of the thermophilic property of celB when being immobilized and determine the optimum temperatures of hydrolysis.

CelB was added to 10 ml of CMC solution (6.7 mg ml^{-1}, pH 5.5, 50 mM acetate) with a concentration of 0.38 μg ml^{-1}. Immobilized celB on PIII treated PS pieces (2 × 4 cm^2) were prepared as described above and added in 10 ml of CMC solution with the same concentration. The tubes were incubated at different temperatures and samples were taken in the linear stage to determine the reaction rate (K, mM min^{-1}) using DNS assay. The absorbance was converted into cellobiose concentration using standard curve from cellobiose. The Arrhenius equation of the hydrolysis is determined as follow:

$$k = A \exp(-E_a/k_B T) \quad (1)$$

where k is the rate constant (min^{-1}), A is a pre-exponential factor, E_a is the activation energy (Joule), $k_B = 1.38 \times 10^{-23}$ J K^{-1} is Boltzmann constant, T is Kelvin temperature.

Rate constants (k) are proportion to the reaction rates (K, mM min^{-1}) due to the same concentrations of substrate used at each temperature. Logarithm values of reaction rates at different temperatures were plotted against $1/T$ to determine the slope of the linear part and subsequently calculate the activation energy

$$\ln K = \ln A - E_a/k_B T \quad (2)$$

2.6 Comparison of free celB and immobilized celB activities

To study the activity of immobilized celB, we compare its V_{max} and K_m values with those of free celB in substrate solutions of increasing concentration. For these comparisons, we have to choose a concentration of free celB which is equal to the amount of immobilized celB on the polymer surface. The concentration of active celB immobilized on PIII treated PS was estimated from the number of molecules to be fitted to the surface area of polymer. If we assume that celB molecules fold in a spherical shape, their minimum radius R_m can be calculated from their mass as proposed by Erickson:[20]

$R_m = 0.066 M^{1/3}$ (M is the molecular weight of protein in Dalton)

CelB has a molecular weight of 118–120 kDa,[13] therefore the minimum radius is approximately 3.25 nm. However, the radius of celB could be larger because celB has 3 domains which are independently folded. Therefore, we chose two concentrations of free celB (0.96 and 0.40 μg ml^{-1}) which correspond to the amount of enzyme in a densely packed monolayer on a

2×4 cm^2 (2 sided) surface assuming enzyme molecular radii 3.1and 4.8 nm respectively. We assumed that dense packing was achieved in a hexagonal lattice (packing density of 0.9068). Ten millilitres of CMC solutions were prepared in 15 ml falcon tubes with concentrations varying from 5 to 50 g l^{-1} in acetate buffer (50 mM, pH = 5). PIII treated polystyrene samples (2×4 cm^2) were incubated with celB overnight and washed as described above before being transferred to the tubes with CMC solution. The experiments using free celB with two concentrations (0.96 and 0.40 μg ml^{-1}) were conducted at the same time. The hydrolysis was performed at 55 °C and 5 samples (300 μl each) were taken from each concentration to determine the reaction rate using the DNS assay. The absorbance at 540 nm was converted into cellobiose concentration by interpolating against the standard curve of cellobiose in the same buffer. The slopes of the straight line which correspond to the reaction rates at each substrate concentration were plotted against CMC concentrations. V_{max} and K_m values were calculated by fitting the points with the following equation:

$$V = V_{max}[S]/(K_m + [S]) \quad (3)$$

where V (mM h^{-1}) is the reaction rate, [S] (g l^{-1}) is the substrate concentration, V_{max} (mM h^{-1}) is the highest reaction rate under the experiment condition, K_m (g l^{-1}) is the Michaelis constant.

2.7 Reuse of immobilized celB

Although the reaction rate increases with increasing temperature, the enzyme tends to be inactivated quicker. To study how the immobilized celB is stable with temperature, the reuse test was conducted with four temperatures (45, 55, 65 and 75 °C). CelB immobilized on PIII treated surface (2×4 cm^2) was added to 10 ml of CMC solution (6.7 mg ml^{-1}, pH 5.5, 50 mM acetate) and incubated in water baths at those temperatures. An aliquot was taken from each tube after 23 hours for DNS assay. After that the PIII treated samples were transferred to new tubes with 10 ml of CMC solution to repeat the hydrolysis. The activity of immobilized celB over 3 courses of re-use was normalized with the first use to compare the activity loss at different temperatures.

2.8 Simultaneously working of celB and β-glucosidase

Recombinant β-glucosidase was cloned and purified as described by Hirsh *et al.*[3] Two sets of experiment were conducted to study the simultaneous hydrolysis of immobilized celB and β-glucosidase on CMC. CelB and β-glucosidase were immobilized on separate PIII treated pieces (1×4 cm^2) in separate containers so as to avoid competition between the two enzymes during covalent immobilisation. The surface immobilised enzymes on PIII supports were then washed and incubated in 10 ml CMC solution (6.7 mg ml^{-1}, pH 5.0, 50 mM acetate) at 55 °C. The surface area ratios of PIII treated PS immobilized with β-glucosidase to PIII treated PS immobilized with celB vary from 0 to 10 in the first experiment with low celB (0.8 cm^2 ml^{-1}) and from 0 to 4 in the second experiment with higher celB (1.6 cm^2 ml^{-1}). An aliquot of 230 μl was taken at 4, 7, 24, 31 and 48 hours and activities of celB and β-glucosidase were determined using the DNS assay and a glucose oxidase/peroxidase assay kit (Sigma #G3660-1CAP) respectively. The reducing sugars as determined by the DNS assay were used to calculate the synergy between immobilized celB and immobilized β-glucosidase. Error bars were estimated at approximately 9.2% due to the total aliquot volume of sampling.

3. Results and discussion

3.1 The use of PIII treatment to achieve covalent surface immobilisation of celB

Fig. 1 shows the subtracted spectra of PIII treated and untreated surfaces before and after treating with SDS. The presence of three amide peaks (3300 cm^{-1} (Amide A), 1650 cm^{-1} (Amide I) and 1540 cm^{-1} (Amide II)) on spectra before SDS indicates the presence of a protein layer on both surfaces after incubation

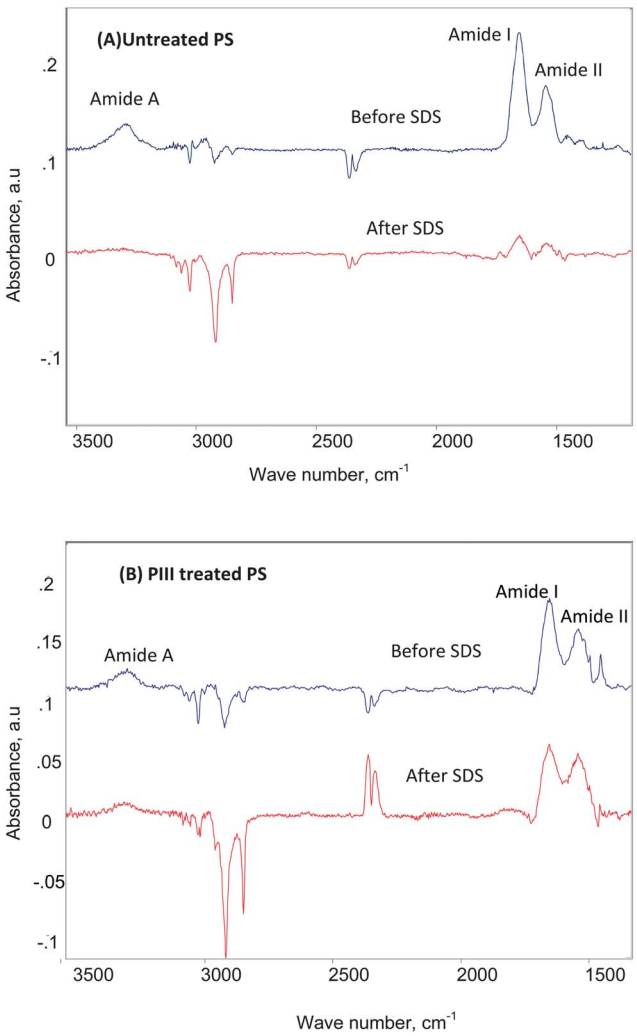

Fig. 1 FTIR-ATR spectra of amide absorptions from immobilised celB on untreated (A) and PIII treated polystyrene (PS) (B) before and after SDS treatment. PS peaks were removed by subtraction of spectra of the same surfaces receiving identical exposure to buffer and atmosphere except without protein. The ability of PIII treated surfaces to retain the enzyme shows covalent binding.

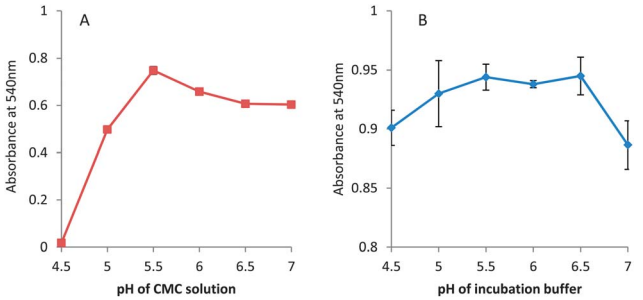

Fig. 2 (A) The dependence of free celB activity on the pH of CMC solution. (B) The dependence of immobilized celB activity on the pH of the solution used for immobilisation.

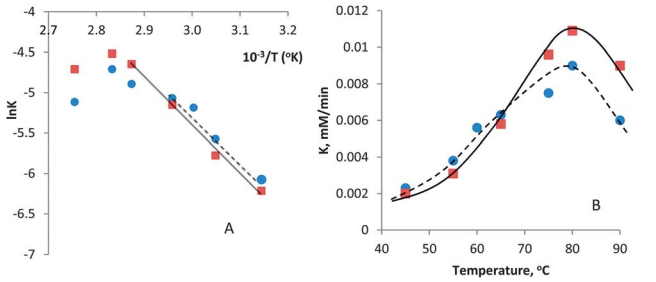

Fig. 3 (A) Plot of logarithm of the reaction rate constant of free celB (■) and immobilized celB (●) as a function of inverse temperature. The linearity of the plot indicates Arrhenius behaviour. (B) Dependence of reaction rate constant for free celB (■) and immobilized celB (●) on temperature. The solid lines are guides for the eye. Temperature induced conformational changes limit the activity at temperatures above 65 °C for immobilised celB and above 75 °C for free celB.

with celB. After the rigorous SDS treatment, there is a significant reduction of amide peak intensities on the untreated surface but only a small reduction on the PIII treated surface. It can be concluded that the PIII treatment greatly increases the ability of the surface to bind the enzyme covalently. The removal of protein from the untreated surface indicates that the binding is mainly by physical adsorption.

3.2 Influence of pH on activity of free and immobilized celB

The activity of free celB in CMC solution is high in the range pH 5–7 and decreases rapidly as the pH decreases below 5 (Fig. 2A). The optimum pH is 5.5 which is similar to the optimum pH of GH5 domain reported by Park *et al.*[14] The pH at which the immobilisation is performed also influences the activity. The relatively high activity of celB (Fig. 2B) after being immobilized in a range of pH buffers from 4.5 to 7 indicates that the orientation of celB relative to the surface and relative to its neighbours when immobilized is favourable so that the active sites of the enzyme are not hindered. The optimum pH range for attachment is 5–6.5. Therefore, pH 5.5 was chosen for all celB immobilisation in this work.

3.3 Thermophilic property of immobilized celB

The temperature dependent behaviour of an enzyme reveals the energetics of its interactions. For an enzyme catalysing a first order reaction with an activation energy of E_a, the rate constant for its catalytic activity increases with temperature according to the Arrhenius relation of eqn (1). The activation energy is determined by the energy barrier to be overcome during the reaction. For an immobilized enzyme it is possible that the proximity of the surface affects the energy barrier. In this experiment, a concentration of celB in solution was chosen to show an activity close to the activity of the immobilized celB on a 2×4 cm^2 PIII treated PS surface. Fig. 3A shows a plot of the logarithm of the reaction rate as a function of inverse temperature and shows Arrhenius behaviour as per eqn (1). The activation energies obtained from the plot of free and immobilized celB are $(8.27 \pm 1.01) \times 10^{-20}$ and $(7.81 \pm 1.36) \times 10^{-20}$ Joule respectively and therefore equal within the uncertainty limits. The similar activation energy of immobilized and free celB implies the immobilization of the celB enzyme molecules on the surface do not substantially affect their activity. There is a departure from the Arrhenius behaviour at 65 °C for the immobilised celB and at 75 °C for the celB in solution. This is attributed to the onset of unfolding which occurs somewhat more readily on the surface. The presence of the surface can destabilise (or lower the energy of some unfolded states of) some enzymes.[21,22] When temperature increases from 40 to 80 °C, the reaction rate of free celB increases 5.5 times as compared to 4.5 times of immobilized celB (Fig. 3B). In both cases, the temperature of 80 °C shows the highest reaction rate. The reaction rates of immobilized celB on the surface are comparable to those of free celB in the middle range (45–65 °C), but are lower at higher temperatures (70–90 °C).

As can be seen from Fig. 3B, the activity of both free and immobilized celB dramatically decreases from 80 to 90 °C. The decrease in reaction rate in this temperature range is similar for free and immobilized celB as partially unfolded lower activity states become dominant.

3.4 Activity of immobilized celB in comparison with free celB in solution

Fig. 4 illustrates the reaction rates as a function of CMC concentration of free celB of two different concentrations

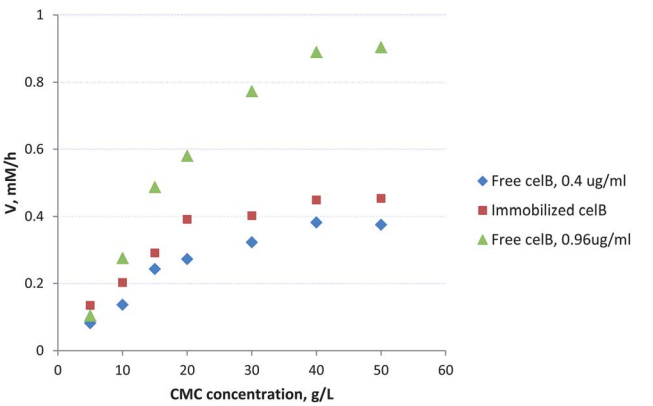

Fig. 4 Enzymatic reaction rate of immobilized and free celB as a function of substrate concentration.

Table 1 V_{max} and K_m values of free and immobilized celB obtained from the fitting of eqn (3) to experimental data

	Free celB 0.4 μg ml^{-1}	Immobilized celB on 16 cm^2	Free celB 0.96 μg ml^{-1}
V_{max}, mM h^{-1}	0.61 ± 0.07	0.64 ± 0.05	1.83 ± 0.35
K_m, g L^{-1}	26.6 ± 6.69	17.33 ± 3.7	45.58 ± 14.88

(0.4 and 9.6 μg ml^{-1}) and immobilized celB. The fitting values of eqn (3) are shown in Table 1. The reaction rate of the immobilised enzyme falls between the values of two different concentrations of free enzyme, allowing a calculation of the effective concentration of the immobilised enzyme. The measured value of 0.48 μg ml^{-1} of this equivalent concentration enables the areal density of active enzyme on the surface to be calculated at 0.3 μg cm^{-2}. This number is comparable to the areal density calculated on the basis of the molecular weight and the assumption of a packing density of 0.9068.

3.5 Reuse of celB immobilized on PIII treated polystyrene (PS)

One advantage of the immobilized enzyme is the possibility of reuse by transferring to a new substrate solution. This avoids inhibition by product accumulation and allows the enzyme to be used for longer times. The results of reuse experiments in which PS surfaces with immobilized celB were moved to a fresh CMC solution every day are shown in Fig. 5 for four different temperatures. The data for each temperature are normalised to the reducing sugars measured after the first use. The amount of reducing sugars detected after 23 hours (first use) at 75 °C is double the amount detected after the same time at 45 °C. The activity of immobilized celB at 75 °C is significantly reduced after the 1st use and no activity remained after the 2nd use (Fig. 5), indicating the complete inactivation of immobilized enzyme on the PIII treated surface. As for samples at 45, 55 and 65 °C, 70%, 60% and 40% of the activity remain after 4 days respectively. Therefore, there is a trade-off between the rate constant and the total activity which should be considered when choosing the optimum working temperature for immobilized celB.

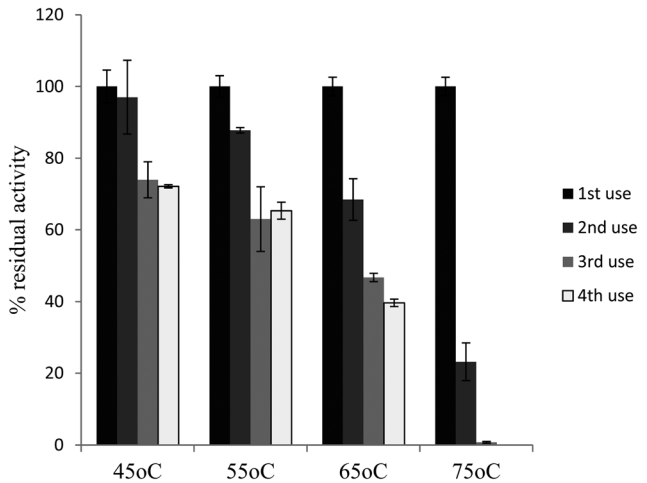

Fig. 5 Comparison of celB activity measured by the DNS assay for reducing sugars for 4 uses (23 hours each) at different temperatures. Activities of the 2nd, 3rd and 4th use were normalized to the activity at the 1st use.

3.6 CMC hydrolysis with immobilized celB and β-glucosidase

CMC is a derivative of cellulose in which hydrogen atoms of the hydroxyl groups on glucose units are replaced by sodium carboxymethyl groups to increase solubility. The substitutions can occur on C_2, C_3 or C_6 of glucose units. The CMC used in this experiment has a molecular weight of 90 000 and a degree of substitution (DS) of 0.7. It was found that with this level of substitution the CMC remains susceptible to enzyme hydrolysis while a DS higher than 1 renders it inert to cellulases.[23] Enzymatic degradation of CMC occurs only between two adjacent unsubstituted anhydroglucose units.[23,24] Therefore the conversion into glucose will become increasingly less complete with increasing degree of substitution.

CelB hydrolyses CMC to produce shorter cello-oligosaccharides and cellobiose. Cellobiose and the smaller cello-oligosaccharides are subsequently hydrolysed by β-glucosidase to produce glucose. Each polymer chain has a reducing end, as do the cellobiose and glucose molecules. The absorbance measured in the DNS assay depends on the total number of cello-oligosaccharide, cellobiose and glucose molecules in solution.

Table 2 shows the total absorbance of reducing sugars in the DNS assay and glucose concentration after 48 hours of the hydrolysis of 67 mg/10 ml of CMC with two different levels of immobilized celB. At high ratios of β-glucosidase (1 : 8, 1 : 10 at a level of 0.8 cm^2 celB per ml and 1 : 4 at a level of 1.6 cm^2 celB per ml), more reducing sugars were produced than by the same amount of celB working alone. This indicates that these high levels of β-glucosidase can reduce cellobiose accumulation and hence, decrease cellobiose inhibition effect on celB activity.

To calculate the enhancement effect of β-glucosidase on the activity of celB, the following ratio was evaluated: the total reducing sugars produced by the enzyme mixture was divided by the sum of reducing sugars produced by celB alone. Note that β-glucosidase acting on CMC alone produces no reducing

Table 2 Reducing end absorbance and glucose concentration of different combinations of immobilized celB and β-glucosidase from 48 hours of CMC hydrolysis at 55 °C

CelB 0.8 cm^2 ml^{-1}						
CelB : β-glucosidase	1 : 0	1 : 2	1 : 4	1 : 6	1 : 8	1 : 10
Reducing end absorbance	1.43	1.37	1.55	1.46	1.7	1.7
Glucose concentration, mg ml^{-1}	0.02	0.12	0.19	0.24	0.26	0.27

CelB 1.6 cm^2 ml^{-1}							
CelB : β-glucosidase	1 : 0	0 : 1	1 : 0.5	1 : 1	1 : 2	1 : 3	1 : 4
Reducing end absorbance	1.80	0	1.80	1.75	1.72	1.84	2.00
Glucose concentration, mg ml^{-1}	0.01	0	0.11	0.20	0.29	0.32	0.36

sugars. The enhancement is dependent on the composition of the mixture and is also expected to be time dependent when one enzyme creates substrate for another. In the case of β-glucosidase and celB, the highest enhancement ratio is 1.19 obtained at the surface area ratios β-glucosidase : celB of 8 and 10. At higher levels of celB, the enhancement obtained at ratio β-glucosidase : celB of 4 is 1.1. These levels of enhancement are small compared to the reported values by Chir *et al.*[25] who studied the synergy between free cel9A and β-glucosidase in CMC hydrolysis using the DNS assay. The CMC concentration (10 mg ml^{-1}) in their study is 1.3 times higher than the concentration used in this study and the concentration of cel9A is 150 times higher than the concentration of free celB which has equivalent activity of 1.6 cm^2 per ml of immobilized celB. In addition, the hydrolysis in Chir *et al.* was conducted at 40 °C with sampling at 1 hour compared to 55 °C and sampling at 48 hours in this experiment. The degree of substitution of carboxymethyl groups on CMC was not specified in the work of Chir *et al.* They reported synergy between the two enzymes at all ratio of the two enzymes tested and the highest synergy was 1.6 at the molar ratio of 1 : 10. The lower enhancement observed in this study could be due to the lower enzyme concentration used or to effects associated with being immobilised on a surface such as reduced accessibility to substrate; hindered access to conformation states used and proximity of the immobilized β-glucosidase and celB to each other. In this case, hindered access to conformation states can be ruled out for celB since the activation energy of the immobilised enzyme is the same as that of the molecule in solution at the temperature for which the synergy study was carried out.

In terms of glucose production, although celB alone can hydrolyse CMC to cellobiose and cello-oligosaccharides, only a very small amount of the monosaccharide, glucose, was detected after 2 days (Table 2). Beta-glucosidase alone cannot hydrolyse CMC; hence no glucose was detected without celB. With a fixed surface area of immobilized celB, increasing surface areas of β-glucosidase resulted in more glucose production. Fig. 6 shows the reaction rates obtained from the slopes glucose concentration *versus* reaction time. The continuous line (from data with 0.8 cm^2 per ml celB) increases quickly as the enzyme area ratio changes from 0 to 6 but begins to saturate between 6 and 10. The discontinuous line (from data with 1.6 cm^2 per ml celB) has the same increasing trend from 0 to 4.

At the same ratio between the two enzymes, the hydrolysis to glucose occurs more rapidly with the higher level of celB (1.6 cm^2 ml^{-1}). For example, when considering the two reactions with the same ratios of enzymes, denoted M in Fig. 6 (1.6 cm^2 per ml of celB and 3.2 cm^2 per ml of β-glucosidase) and N (0.8 cm^2 per ml of celB and 1.6 cm^2 per ml of β-glucosidase), while the surface areas of both enzymes increase by a factor of two, the reaction rate triples indicative of the synergy between the two enzymes. Apart from hydrolysing cellobiose, β-glucosidase from *Caldicellulosiruptor saccharolyticus* has been reported to show activity on cellotriose, cellotetraose and cellopentaose.[26] The high level of celB produces more short chain cello-oligosaccharides which in addition to cellobiose are also substrates

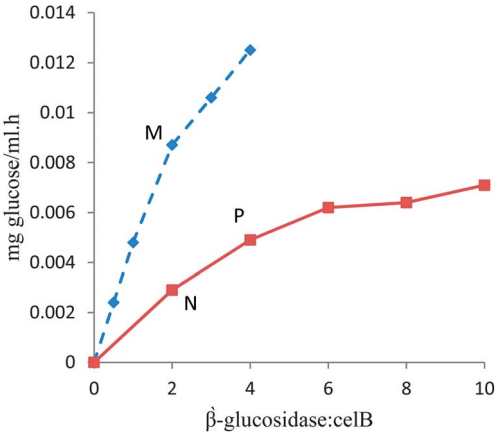

Fig. 6 Influence of ratios between β-glucosidase and celB on glucose production. The continuous line represents initial reaction rates (as determined by the slope of glucose curves in Fig. 5B and D) of experiments with 0.8 cm^2 per ml celB and the discontinuous line represents reaction rates of experiments with 1.6 cm^2 per ml celB. The points M, N and P are discussed in the text.

for β-glucosidase. Therefore, with the same surface area of immobilized β-glucosidase – Fig. 6 points P (0.8 cm^2 per ml of celB and 3.2 cm^2 per ml of β-glucosidase) and M (1.6 cm^2 per ml of celB and 3.2 cm^2 per ml of β-glucosidase) – an increase in celB resulted in a higher glucose production rate.

Enzyme loading can be readily increased by increasing the area of polymer surfaces with immobilized enzyme. Since only a monolayer of enzyme can be covalently attached to the surface, the enzyme concentration can be maximised by maximising the total area of immobilization surfaces in the reactor volume whilst taking care to ensure that substrate from solution can easily access all of the surfaces. Beads can be used to significantly increase the available surface area in a given volume, increasingly so for small diameter beads. Bead geometry would also be more favourable for the accessibility of substrate to the immobilized enzyme reducing substrate depletion near the surface. The enhancement effect may also be increased with a fairly homogenous distribution of small sized beads. A homogenous distribution of celB beads and β-glucosidase beads will reduce the travel distance of substrate–product exchange between celB-bead surfaces and β-glucosidase bead surfaces. The benefit of large surface area of nano/microsized hybrid materials in immobilizing enzymes has been reviewed.[12,27] The enhancement of distribution between enzyme carriers and the substrate becomes more important when insoluble cellulose is used as has been demonstrated from the immobilization of cellulases and hemicellulases on 2 μm glass porous beads.[28]

4. Conclusion

This paper shows that celB and β-glucosidase function effectively together to produce glucose from CMC when covalently immobilised on PIII treated PS. Recombinant celB is optimally immobilised in a pH range 5–6.5 and exhibits a maximum activity when used at pH 5.5. Immobilized celB shows a similar

activation energy to the free enzyme in CMC hydrolysis. The areal density of immobilized celB is estimated to be 0.3 μg cm^{-2}. Immobilized celB retained 70% of its initial activity after 4 uses of 23 hours each. High ratio of immobilized β-glucosidase enhances the activity of immobilized celB in CMC hydrolysis.

Acknowledgements

The authors would like to thank Serve Kengen for provision of the Genomic DNA from *Caldicellulosiruptor saccharolyticus*. Many thanks are expressed to Professor Cris dos Remedios for stimulating discussions and for access to laboratory facilities used in this study. This work is financially supported by the Australian Research Council.

References

1 Novozymes, http://ethanolproducer.com/articles/8580/novozymes-announces-new-advanced-biofuels-enzyme-technology.
2 Genencor, http://biosciences.dupont.com/fileadmin/user_upload/genencor/documents/AccelleraseTRIOProductLiterature_120719.pdf.
3 S. L. Hirsh, N. J. Nosworthy, A. Kondyurin, C. G. dos Remedios, D. R. McKenzie and M. M. M. Bilek, *J. Mater. Chem.*, 2011, **21**, 17832–17841.
4 M. M. M. Bilek, D. V. Bax, A. Kondyurin, Y. Yin, N. J. Nosworthy, K. Fisher, A. Waterhouse, A. S. Weiss, C. G. dos Remedios and D. R. McKenzie, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 14405–14410.
5 A. Kondyurin, P. Naseri, K. Fisher, D. R. McKenzie and M. M. M. Bilek, *Polym. Degrad. Stab.*, 2009, **94**, 638–646.
6 E. A. Kosobrodova, A. V. Kondyurin, K. Fisher, W. Moeller, D. R. McKenzie and M. M. M. Bilek, *Nucl. Instrum. Methods Phys. Res., Sect. B*, 2012, **280**, 26–35.
7 D. V. Bax, Y. Wang, Z. Li, P. K. M. Maitz, D. R. McKenzie, M. M. M. Bilek and A. S. Weiss, *Biomaterials*, 2011, **32**, 5100–5111.
8 A. Kondyurin, N. J. Nosworthy and M. M. M. Bilek, *Acta Biomater.*, 2008, **4**, 1218–1225.
9 C. MacDonald, R. Morrow, A. S. Weiss and M. M. M. Bilek, *J. R. Soc., Interface*, 2008, **5**, 663–669.
10 C. T. Tran, A. Kondyurin, S. L. Hirsh, D. R. McKenzie and M. M. Bilek, *J. R. Soc., Interface*, 2012, **9**, 2923–2935.
11 A. V. Kondyurin, P. Naseri, J. M. R. Tilley, N. J. Nosworthy, M. M. M. Bilek and D. R. McKenzie, arXiv:1110.3125 2011.
12 E. T. Hwang and M. B. Gu, *Eng. Life Sci.*, 2013, **13**, 49–61.
13 D. J. Saul, L. C. Williams, R. A. Grayling, L. W. Chamley, D. R. Love and P. L. Bergquist, *Appl. Environ. Microbiol.*, 1990, **56**, 3117–3124.
14 J. I. Park, M. S. Kent, S. Datta, B. M. Holmes, Z. Huang, B. A. Simmons, K. L. Sale and R. Sapra, *Bioresour. Technol.*, 2011, **102**, 5988–5994.
15 J.-D. Bok, D. A. Yernool and D. E. Eveleigh, *Appl. Environ. Microbiol.*, 1998, **64**, 4774–4781.
16 A. Kondyurin and M. Bilek, in *Ion beam treatment of polymers*, Elsevier, Amsterdam, 2008.
17 Y. Yin, M. M. M. Bilek, D. R. McKenzie, N. J. Nosworthy, A. Kondyurin, H. Youssef, M. J. Byrom and W. Yang, *Surf. Coat. Technol.*, 2009, **203**, 1310–1316.
18 C. T. H. Tran, A. Kondyurin, W. Chrzanowski, M. M. M. Bilek and D. R. McKenzie, *Colloids Surf., B*, 2013, **104**, 145–152.
19 G. L. Miller, *Anal. Chem.*, 1959, **31**, 426–428.
20 H. P. Erickson, *Biol. Proced. Online*, 2009, **11**, 32–51.
21 D. B. Berkowitz and D. W. Webert, *J. Immunol. Methods*, 1981, **47**, 121–124.
22 J. D. Whittle, N. A. Bullett, R. D. Short, C. W. Ian Douglas, A. P. Hollander and J. Davies, *J. Mater. Chem.*, 2002, **12**, 2726–2732.
23 R. A. Gelman, *J. Appl. Polym. Sci.*, 1982, **27**, 2957–2964.
24 M. G. Wirick, *J. Polym. Sci., Part A-1: Polym. Chem.*, 1968, **6**, 1965–1974.
25 J. Chir, C. Wan, C. Chou and A. Wu, *Biotechnol. Lett.*, 2011, **33**, 777–782.
26 M.-R. Hong, Y.-S. Kim, C.-S. Park, J.-K. Lee, Y.-S. Kim and D.-K. Oh, *J. Biosci. Bioeng.*, 2009, **108**, 36–40.
27 M. Verma, C. Barrow and M. Puri, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 23–39.
28 P. Mandali and B. K. Dalaly, *J. ASTM Int.*, 2010, **7**, DOI: 10.1520/JAI102580.