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Kinetic Hydrolysis of Cellulose Biopolymer by Carbon Nanotubes Immobilized Cellulase

Nurul Dayana Azmi[1], Muhammad Naziz Saat[2,*], Mohamad Suffian Mohamad Annuar[1]

[1] Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Wilayah Persekutuan, Malaysia
[2] School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Allam, Selangor, Malaysia

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ABSTRACT

Immobilized enzymes are widely used in various biochemical reactions due to higher stability and reusability. Immobilization enzyme is a process of confinement enzyme molecules onto/within a support or matrix material *via* the chemical or physical process. In this study, the effect of free and CNT-Immobilized cellulase was investigated for hydrolysis of different CMC concentrations. The effect of free and CNT-Immobilized cellulase on the kinetic parameters V_{max} and K_m in the CMC hydrolysis was compared. The maximum reaction velocity V_{max} of CNT-Immobilized cellulase is similar to free cellulase which indicates cellulase adsorption in CNT is effective in hydrolyzing CMC. However, the observed K_m values for CNT-Immobilized cellulase is higher than free cellulase for different CMC concentrations. The K_m value for CNT-Immobilized shows a sharp decrease in substrate affinity as compared to free cellulase due to diffusional restrictions by the CNT matrix. In addition, the initial velocity (V_0) of CMC hydrolysis by CNT-Immobilized cellulase shows similar increasing pattern with the increase of reaction mixture viscosity. The current preparation of CNT-Immobilized cellulase have almost the same catalytic reaction in CMC hydrolysis as free cellulase. Thus, the current preparation of CNT-Immobilized has significant potential for green and sustainable biocatalyst in cellulose-based biopolymer hydrolysis reaction.

1. Introduction

Carboxymethyl cellulose (CMC) is a derivative of natural cellulose polymer composed of glucopyranose monomer units as the main backbone of the polymer. The main structure of CMC is characterized by the carboxymethyl groups (-CH_2COOH) groups link to the hydroxyl groups (-OH) of glucopyranose monomer unit at cellulose backbone. Cellulose is considered as the most abundant type of polysaccharide in nature and has been classified as a renewable source to produce biofuels. Degradation of cellulose polymer occur in the presence of water molecules for each monomer of cellulose. Cellulose can be found in plants as lignocellulose complex associated with lignin. Lignin is distinctively characterized by their structural rigidity compound which resistant against enzymatic

* *Corresponding author.*
E-mail address: naziz@uitm.edu.my

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and acidic molecules. Thus, CMC degradation is considered as slow process as the efforts to develop an economically feasible hydrolysis system have been hampered due to low conversion rate.

Application of immobilized enzyme have received much attention due to several advantages including easy product recovery, excellence protection at extreme temperature and pH [1,2]. On the other hand, the immobilized enzymes can be used repetitively and continuously, and maintain their biocatalytic activity with more stability than mobile enzymes [3,4]. Cellulase enzyme has been immobilized on various carrier materials including silicate materials [5], alginate beads [6], chitosan microspheres [7], nano-particles [8], and copolymers [9]. Recently, carbon nanotubes (CNT) have received significant interest as carrier material for enzyme immobilization due to its outstanding properties in thermal, chemical, and mechanical resistances [10]. The preparation of carbon nanotubes is carried out by using either multi-walled carbon nanotubes or single-walled carbon nanotubes.

As reported, multi-walled CNT are preferred over single-walled CNT for application in enzyme immobilization in favour of their simple preparation and cost effectiveness. The multi-walled CNT is considered superior in its tensile strength of structural arrangement rather than single-walled CNT [11,12]. Immobilizing cellulase on the multi-walled CNT increases the thermal stability, makes enzymatic recycling easier and the catalytic activity can be easily controlled [13]. Studies of enzyme immobilization using CNT have shown the significant application of multi-walled CNT in numerous enzymes such as catalase [14], cellulase [15], horseradish peroxidase [16], inulinase [17], laccase [18], lipase [19], papain [20], phenylalanine ammonia lyase [21], pyranose oxidase [22], xylanase [23], and β-galactosidase [24].

In the present study, multi-walled carbon nanotubes was used in the immobilization of cellulase from *Aspergillus niger* in carboxymethyl cellulose hydrolysis. Different concentrations of carboxymethyl cellulose (CMC) were hydrolyzed by free and immobilized cellulase and their kinetic parameters of maximum reaction velocity (V_{max}) and Michaelis constant (K_m) was calculated. In addition, the effect of viscosity from different CMC concentrations on the kinetic parameters of CMC hydrolysis was investigated.

2. Methodology

2.1 Materials

Commercial cellulase (EC 3.2.1.4) from *Aspergillus niger* (powder, ≥ 0.3 units/mg solid; Cat No: C1184), multi-walled carbon nanotubes (MWCNT, Cat No:773840-25G), 3,5-Dinitrosalicylic acid (DNS, Cat No: D0550-100G), Glutaraldehyde solution (Grade II 25%, Cat No: G6257-100ML) were purchased from Sigma-Aldrich. The components of hydrolysis reaction medium such as substrate Carboxymethyl Cellulose (CMC, Cat No: C5678-500G) and buffer sodium acetate (Cat No: 241245-500G), are of analytical or ACS reagent grade. DNS Reagent preparation including liquified phenol (Cat No: P9346-100ML), sodium hydroxide (Cat No: S5881-500G), sodium sulphite (Cat No: 239321) are purchased in the form of ACS reagent or analytical grade. All aqueous solutions was prepared using ultrapure water (18.2 MΩ cm resistivity) obtained from an ultrapure water purifying system (Heal Force®, Shanghai, China).

2.2 Carbon Nanotube Immobilization of Cellulase

Carbon nanotubes suspension was prepared *via* ultrasonication by adding 200 mg solid powder of MWCNT in 50 ml of acetate buffer (50 mM, pH 4.7) for 30 min using a batch sonicator (Elmasonic P30H, Elma, Singen, Germany) with 0.8 W sonication power at 37 kHz. About 10.0 mL of cellulase

enzyme (20 Unit/mg activity) and 0.3 % (*v*/*v*) glutaraldehyde was added into MWCNT suspension before subjected to mixing *via* shaking incubator (200 rpm at room temperature). Then, the mixture was centrifuged for 10 minutes at 8000 rpm in order to discard the supernatant from the pellets of CNT-Immobilized cellulase. For immediate usage, pellets was washed three times with acetate buffer prior adding into the reaction medium. The remaining pellets was kept in a seal container with small amount of acetate buffer at 4 °C for maximum 3 days in order to avoid loss of enzyme activity during storage.

2.3 Batch Hydrolysis of Carboxymethyl Cellulose

Batch hydrolysis reaction took place in 100 ml glass bottle (Brand: Schott, Germany) consisted of 20 ml of acetate buffer (pH 4.70), 20.0 ml of substrate carboxymethyl cellulose, CMC (2.5, 5.0, 7.5, 10.0 and 12.5 mg L^{-1}) and 10.0 ml of CNT-Immobilized cellulase suspension. The glass bottle was screwed on with a cap and sealed with PTFE tape to minimize evaporation of reaction mixture. The condition for batch reaction was carried out using shaking incubator (Daihan, China) with agitation speed of 150 rpm at controlled temperature of 40 °C. About 2.0 mL of samples was withdrawn from the batch reaction at regular intervals for reducing sugar analysis.

2.4 Estimation of Glucose Concentration

Glucose concentration was determined *via* dinitrosalicylic acid (DNS) analysis according to Miller [25]. About 1.5 mL of DNS reagent was added into a test tube contain sample of 0.5 ml followed by incubation in hot water bath at 90 °C for 15 minutes. Then, 0.5 mL of Rochelle salt solution was added into the mixture of DNS reagent and sample in order to stabilize the colour changes, indicates the present of reducing sugar product. After cooling down to room temperature, the DNS reagent and sample mixture was subjected to spectrophotometric analysis at 575 nm. The actual amount of reducing sugar formation was calculated *via* standard calibration plot of absorbance and different known glucose anhydrous concentrations.

2.5 Viscosity Measurement

A vibrational viscometer (SV-10 Brand: A&D) was used to measure sample viscosity. The viscometer was filled with fresh reaction mixture until the oscillator was fully submerged and allowed to equilibrate for 5 minutes before the viscosity measurement was recorded in 3 replicates for each CMC concentrations.

2.6 Enzyme Assay

Enzyme assay determination was carried out with 1 mL enzyme solution was added into reaction mixture of 2.0 mL acetate buffer (0.05 M, pH 4.8) and 2.0 mL of CMC salt solution (0.1 % w/v). The mixture was incubated in seal test tube at 40 °C, for 20 minutes. DNS assay was used to determine the reducing sugar analysis according to Miller [25]. By definition, one unit of enzyme activity (1 U) was the required amount of enzyme during the liberation of 1.0 µmol of reducing sugars from cellulose substrate per minute at 40 °C. The calculated enzyme activity was determined from Eq. (1) where *E* is the enzyme activity (U) and t is the total reaction time (min).

$$E(\mathrm{U}) = \frac{\mu \times \mathrm{mol}_{\text{Reducing sugar released}}}{t_{\mathrm{min}}} \quad (1)$$

2.7 Initial Rate and Kinetic Model

Initial rate of reaction was calculated *via* tangent of origin on the time profile of liberated reducing sugars from CMC hydrolysis. Calculated initial rate (V_0) was used to fit the Michaelis-Menten model as written in Eq. (2). The kinetic parameters of Michaelis-Menten model of CMC hydrolysis reaction namely Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) was determined by using linearization method of double reciprocal plot of Lineweaver-Burk plots as written in Eq. (3). Curve fitting of Michaelis-Menten model on the experimental data of CMC hydrolysis was done by using Polymath 6.0.

$$V_0 = \frac{V_{max} \times K_m}{K_m + S} \tag{2}$$

$$\frac{1}{V_0} = \frac{K_m}{V_{max}(S)} + \frac{1}{V_{max}} \tag{3}$$

3. Results

3.1 Hydrolysis Reaction

Cellulase from *Aspergillus niger* was preferred as biocatalyst due to its superior properties as compared to other strains and has been frequently used in industry [26, 27]. In this study, free and immobilized system were employed for the CMC hydrolysis reaction by Cellulase. Reaction condition was carried out at 40°C with different agitation speed of 200 rpm. Figure 1 shows the glucose production profile of CMC hydrolysis reaction using free and immobilize cellulase. Glucose production in both free and CNT-Immobilized cellulase shows increasing profile until 2 minutes incubation for the majority CMC concentrations before glucose production is constant thereafter. In addition, the accumulation of glucose causes subsequent drop of cellulase activity due to the reduction in reaction velocity which is common in the batch reaction [28,29]. However, reduction in reaction velocity is often not observable in this study since the reaction thermodynamics favours product formation [30].

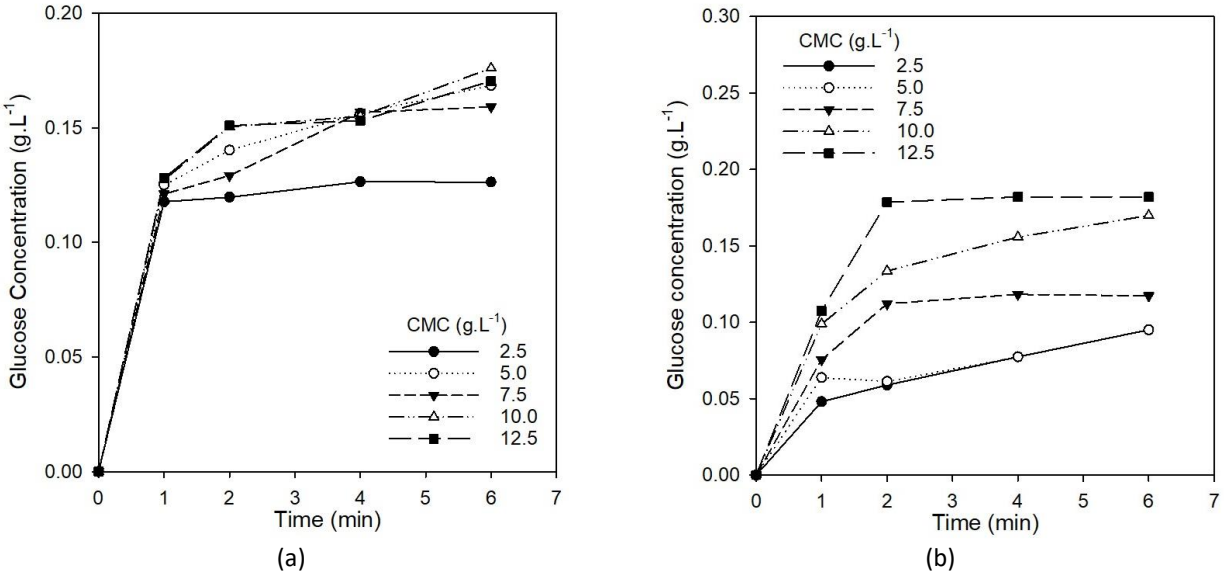

Fig. 1. CMC hydrolysis profile (a) Free enzyme (b) CNT-immobilized

3.2 Enzyme Kinetic Parameters

The effect of enzyme immobilization using carbon nanotube (CNT) in the glucose production profile is not clearly observed due to large variation in the typical time courses of CMC hydrolysis reaction. To validate the effect of CNT-Immobilized in CMC hydrolysis reaction, the kinetic parameters including K_m and V_{max} are determined by using non-linear regression analysis by Sigma Plot Software. Figure 2 showed the double reciprocal plot of both free and CNT-Immobilized cellulase for hydrolysis reaction of different CMC concentrations.

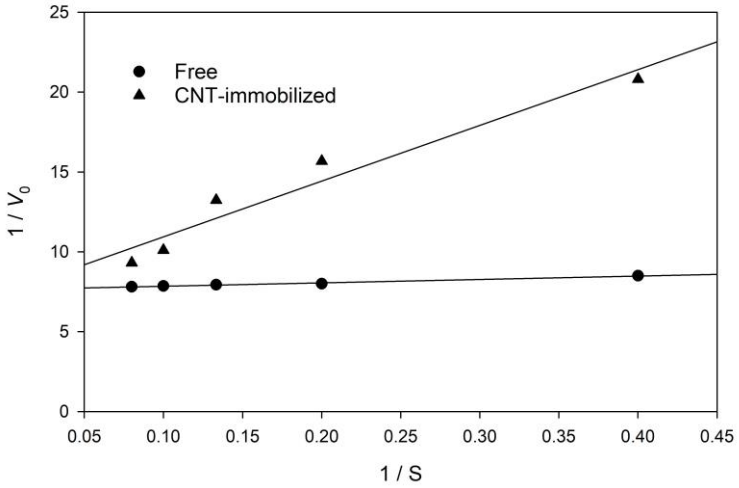

Fig. 2. Double reciprocal plot for free and CNT-Immobilized cellulase for CMC hydrolysis reaction

The apparent kinetic parameters of Michaelis constant (K_m) and maximum reaction velocity (V_{max}) for glucose production in both free and CNT-Immobilized cellulase was summarized in Table 1. Coefficient correlation (R^2) for both free and CNT-Immobilized cellulase were 0.9869 and 0.9451, respectively, indicating better prediction of both kinetic parameters on fitted experimental data [31]. The V_{max} of both free and CNT-Immobilized cellulase showed similar values due to the direct contact of substrates and cellulase active site since the enzyme was immobilized on the surface of CNT [32]. From this study, K_m of CNT-Immobilized cellulase is higher than free cellulase. Thus, higher enzyme concentration is required for the immobilized system to reach maximal reaction velocity [33]. In addition, difference in K_m values is attributed with the diffusion limitation in the CNT-Immobilized cellulase. As compared to CMC hydrolysis using free cellulase, impact of diffusional limitation in the CNT-Immobilized was observed as K_m value increase. Substrate molecules have to diffuse from bulk solution into cellulase active site while overcoming the matrix of CNT. It can be assumed the rate of diffusion in CNT-Immobilized cellulase is slower than the rate of transformation since more substrate is needed for the reaction to reach maximal reaction velocity [34]. In addition, the stearic effects of CNT molecules is associated with the changes in the catalytic activity between cellulase active site and substrate as the reason of increase in K_m value [35].

Table 1
Kinetic parameters for free and CNT-Immobilized Cellulase in CMC hydrolysis reaction

Parameter	Free Cellulase	CNT-Immobilized
K_m (g L^{-1})	0.2794	4.6810
V_{max} (g L^{-1} min^{-1})	0.1311	0.1342

3.3 Effect of Viscosity

According to viscosity analysis of reaction mixture with different CMC concentrations, there was an increase of initial velocity of glucose production with increasing viscosity values. At higher viscosity, substrate needs to overcome the mass transfer limitation in the viscous reaction mixture due to different CMC concentrations [36,37]. At high CMC concentrations, the presents of soluble cellulose fibers increased viscosity by means of 10-fold for 1% concentrations [38]. Despite an increase of viscosity in the current study, initial velocity (V_0) remains increased with CMC concentrations (Figure 3). This observation could be due to sufficient mixing in the reaction mixture at agitation speeds of 200 rpm which negates the hindering effect of increased viscosity.

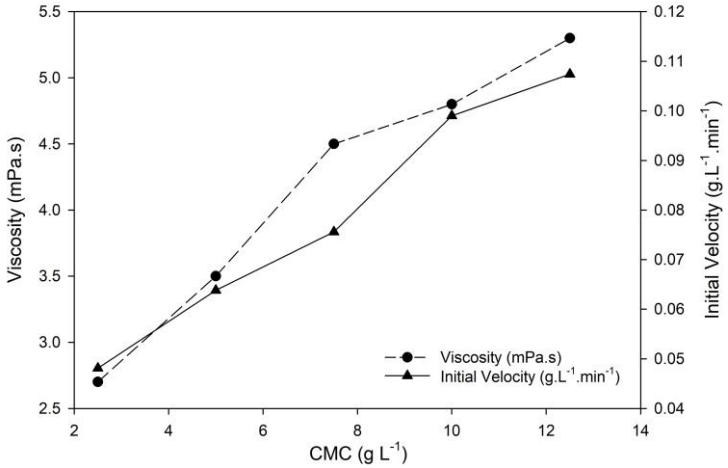

Fig. 3. Effect of viscosity on initial velocity V_0 of CNT-Immobilized cellulase for different CMC concentrations

4. Conclusions

In conclusion, the catalytic activity of CMC hydrolysis by CNT-Immobilized cellulase can approach similar to free cellulase during the production of reducing sugar. The maximum reaction velocity (V_{max}) of CMC hydrolysis by free cellulase and CNT-Immobilized cellulase was 0.1311 and 0.1342 g L^{-1} min^{-1}, respectively. However, the effect of CNT-Immobilized on the Michaelis constant (K_m) was higher at 4.6810 g L^{-1} as compared to free cellulase with K_m of 0.2794 g L^{-1}. The diffusion limitation in high viscous solution increased the Michaelis constant value during the CMC hydrolysis reaction. The incorporated of cellulase onto CNT molecules is an innovative technique in nanobiotechnology applications from an economic point of view since it can be easily separated from the end product and reused for multiple times.

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