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A KINETIC STUDY OF ENZYMATIC HYDROLYSIS OF OIL PALM BIOMASS FOR FERMENTABLE SUGAR USING POLYETHYLENE GLYCOL (PEG) IMMOBILIZED CELLULASE

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Graphical abstract

Parameter	Free cellulase	Immobilized cellulase
Michaelis constant (K_m), mg/ml	171.8	179.2
Limiting velocity (V_{max}), mg/ml.min	34.5	33.5

Abstract

In this work, enzymatic hydrolysis by cellulase in a soluble and an immobilized form was studied to convert lignocellulosic oil palm empty fruit bunch (EFB) biomass into fermentable sugars as a feedstock for bioethanol production. The cellulase was covalently immobilized with activated and functionalized polyethylene glycol (PEG) via glutaraldehyde coupling method. As a whole, the immobilized cellulase displayed 50% higher efficiency over free cellulase, in reducing sugar recovery during hydrolysis reactions at pH of 4.8 and temperature of 50°C. From the kinetic study, it showed that Michaelis constant (Km) and limiting velocity (V_{max}) of immobilized cellulase were 179.2 mg/ml and 33.5 mg/ml.min respectively, comparable with the value for free cellulose, 171.8 mg/ml and 34.5 mg/ml.min respectively. This result could be attributed to the effect of PEG on the binding cellulase to substrate desorb substrates, and enables free interaction of cellulase to hydrolyse cellulose maximally.

Keywords: Oil Palm Empty Fruit Bunch (EFB); hydrolysis; fermentable sugars; immobilized cellulase; Glutaraldehyde Coupling Method.

Abstrak

Didalam kajian ini, enzim hidrolisis menggunakan selulase didalam keadaan larut dan pegun telah digunakan untuk menukarkan lignoselulosa tandan kelapa sawit kepada gula mudah-tapai sebagai stok suapan untuk penghasilan bioetanol. Selulase telah dipegunkan secara kovalen bersama polietilene glycol (PEF) yang teraktif melalui kaedah gandingan glutaraldehid. Secara keseluruhan, selulase pegun mempamerkan 50% kecekapan tinggi atas selulase bebas, dalam pemulihan gula penurun semasa tindak balas hidrolisis di pH 4.8 dan suhu 50°C. Kajian kinetik, menunjukkan bahawa Michaelis malar dan hadlaju bersempadan bagi selulase pegun ialah 179.2 mg / ml dan 33.5 mg / ml.min , setanding dengan nilai yang diperolehi daripada selulase bebas ,171.8 mg / ml dan 34.5 mg /ml. Keputusan ini mungkin disebabkan oleh kesan pengikatan PEG dengan selulase yang menyahjerap substrat, dan membolehkan interaksi bebas hydrolisis selulase kepada selulosa secara maksimum.

Kata kunci: Tandan Kosong Buah Minyak Kelapa Sawit (EFB); hidrolisis ; gula beragi ; bergerak selulase ; Kaedah gandingan Glutaraldehida.

1.0 INTRODUCTION

The recent attention in cellulosic biomass conversion to bioenergy and useful chemicals is due to its potentials and abundances. Cellulosic biomass refers to all natural plant and shrub matter containing high carbohydrate content i.e. agricultural and forest residues, industrial and municipal wastes. They are otherwise called as a second generation feed stocks for biofuel production, which being alternative to food biomass sources (i.e cassava, wheat etc) that pose threat to food security [1]. Structurally, cellulosic biomass is a composite of cellulose chains which held together by hydrogen bonding, and its fibers are interlock with hemicellulose and lignin. The components of cellulose and hemicellulose are part of polysaccharides that can be hydrolysed, either with the aid of acid or enzyme, into reducing sugars, which can be fermented or chemically transformed into valuable fuels and chemicals [2].

The method of cellulose hydrolysis is mainly determined by the recalcitrant and crystalline structural of the biomass lignocellulosic [3]. And this has become a challenge on the commercialization of biofuel production from the biomass resource. Therefore, appropriate biomass pretreatment to depolarize its structure and ease its accessibility to enzyme during hydrolysis process for targeted product is very important. Kumar *et al*, (2009) reported that, since pretreatment commence bioethanol process from lignocellulose biomass; its efficiency has a direct effect on the subsequent steps [4]. In that case, the following factors must be considered for high-efficiency pretreatment: i) high yield from feedstock, ii) high sugar concentration, iii) the increase of enzyme and fermentation compatibility, iv) minimum cost and v) Ability to scale to commercial production [4].

The pretreatment method of lignocellulose biomass to enhance its solubilisation into fermentable sugars, can be broadly classified into four types: i) physical methods (e.g., milling and grinding); ii) physiochemical methods (e.g., steam explosion or hydrothermolysis); iii) chemical methods (e.g., using acids, alkali, oxidizing agents, or organic solvents to treat biomass); and iv) biological methods (e.g., using bacteria and fungi to treat biomass) [5]. Subsequently, in the hydrolysis process, the carbohydrate polymers are broken into fermentable sugars with the aid of either acid or enzymes. The acid process is not a convenient process due to its corrosiveness and it leads to the formation of toxic products that can interfere with fermentation [6].

Research findings have shown that, enzymatic hydrolysis is one of efficient means of obtaining fermentable sugars from biomass. In the cellulase system, which is a combination of some hydrolytic enzymes, is required to hydrolyse cellulose into glucose monomers. Cellulase is an enzyme produced majorly by fungi and bacteria. This system of enzymes acts synergistically to hydrolyse cellulose by its β-glycosidic linkages. Enzyme substrate complex is formed during hydrolysis with the influence of endoglucanase and cellobiohydrolase on bonds. Endoglucanase breaks β-glycosidic bonds at varied locations on the cellulose chain through the catalytic domain (CD). Then the free chain end of cellulose is converted into cellobiose units by cellobiohydrolase, while β- glucosidase breaks the β-glycosidic bond between the glucose dimer, resulting into glucose monomers [7]. However, to obtain desired output from enzymatic hydrolysis, enzyme to be used should be immobilized with compatible and efficient support via convenient and efficient method. This is because, generally, enzymes are relatively unstable, their cost of isolation is high, and it is very challenging to recover the active enzyme after use [8].

The materials that could be used as an enzyme immobilized support can be classified as organic support and inorganic support-depending on their chemical composition. The organic supports are majorly classified into natural and synthetic polymers. The organic supports are mostly used in industrial applications, because this hydrophilic character is a prerequisite in determining the level of immobilized enzyme activity [9]. For that reason, polyethylene glycol has been chosen as support for immobilization of cellulase in this research work due to its good hydrophilic property. In addition, polyethylene glycol (PEG) is obtained at low cost and it has useful properties like biological compatibility, and ease of chemical modification.

When immobilizing an enzyme to a surface, it is most important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of the enzyme [10]. The common methods of enzyme immobilization are: covalent coupling, adsorption, cross-linking and entrapment. Although each of the aforementioned methods of immobilizing enzymes has a peculiar setback but covalent binding are generally stronger due to the firm bonding between the enzyme and the support. The covalent bonding is also can improve the stereospecificity of the immobilized enzymes as well as thermal stability and bioactivity [11]. For better assessment of immobilized enzymes, its kinetic influence on the hydrolysis reaction needs to be evaluated. Several researches have been done on kinetic study of immobilized cellulase with so many carriers, but polyethylene glycol has not been reported. As such, this research work aims at kinetic study on enzymatic hydrolysis of oil palm empty

fruit bunch into fermentable sugars using PEG immobilized cellulase.

2.0 MATERIALS AND METHODS

2.1 Raw Material

The empty fruit bunch sample was obtained from oil palm mill located in Simpang Waha, Kota Tinggi, Johor, Malaysia. Cellulase Cellulase From Aspergillus niger, powder, >0.3 units/mg solid (Cas No. : 9012-54-8) was purchased from Sigma Aldrich. The chemicals used, standard glucose (D(+)-glucose monohydrate), citrate buffer, PEG-6000, sodium azide, 3,5-dinitrosalicylic acid (DNS), sodium potassium tartarate, acetic acid, citric acid monohydrate, sodium borohydride, and sodium chlorite were purchased from Sigma-Aldrich. All the reagents used were of analytical grade.

2.2 Preparation of 3-5-Dinitrosalicylic Acid (DNS) Reagent

An amount of 1.0 g of 3,5-dinitrosalicylic acid (DNS) was dissolved in 30 ml of distilled water. Then, 30 g of sodium potassium tartarate was added to the solution. Subsequently, 20 ml of 2N sodium hydroxide was added to the mixture until it turned to transparent orange yellow in colour. Finally, the solution was made up to 100 ml with distilled water and stored in an amber coloured bottle.

2.3 Preparation of Standard Glucose Solution

Stock standard glucose solution was prepared by dissolving 1.5 g of D(+)-glucose monohydrate in 100 ml volumetric flask and stirred gently for easy dissolution. Thereafter, the different concentrations of standard glucose solution ranging from 0.3 to 1.5 mg/ml were prepared in six test tubes. The test tubes were heated in boiling water for 5 minutes. DNS reacted with the glucose (reducing sugar) and turned from yellow to red/brown colour, because of its conversion to 3-amino, 5-nitrosalicyclic acid. After the test tubes have cooled, 6 ml of distilled water was added to each of them and shaken well, before taking their respective absorbance at 540 nm using Perkin Elmer UV-Vis spectrophotometer (Lambda 25).

2.4 Steam Explosion Pretreatment Sample.

In this study, the steam explosion was employed for biomass pretreatment as the method is free from chemical usage that can affect the enzyme, and due to its simplicity. In this process, the obtained empty fruit bunch (EFB) was washed with distilled water and dried under the sun for two days. Thereafter, the sample was sliced and screened with laboratory test sieve with 1.0 mm aperture. 50g of EFB was introduced into the bottle containing 400ml distilled water and heated in a pressure cooker unit (All American 921) with two-third level of water at 190° and 10 bar for 30 minutes. After the designated cooking time has elapsed, an exploded sample (EFB) was collected in 200ml beaker dried at 50°C in an oven (Memmert-model) overnight and stored for subsequent used.

2.5 Immobilized of Cellulase With PEG as Carrier

The procedure of immobilized the cellulose with PEG was conducted based on little modification on the procedure of Divya et al. (1998) [12]. An amount of 0.25 g of PEG (PEG) was introduced in to 250 ml Erlenmeyer flask containing 10 ml of 0.1M of nitric acid and 0.1 M of sulphuric acid in ratio 1 to 3, and heated at 75°C for 2 hours. The solubilized PEG was neutralized with the addition of 1 ml of 0.5 M sodium hydroxide. Then, 0.3 g of sodium borohydride was added to the PEG solution and incubated overnight at room temperature, in order to convert the associated nitrite group of PEG to amine group. A solution of 5 ml of 2.5 % (v/v) of glutaraldehyde was added to the PEG mixture and left to react for 30 minutes at 4°C. Afterwards, the pH value of the solution was adjusted from 10 to 5.6, by adding citrate buffer with the total volume of 20 ml. Then 2 ml of cellulase was added to the solution, shaken with incubator shaker at room temperature, 150 rpm for 2 hour and later left to react at 4°C for 24 hours. The pH value of the mixture was adjusted to 5 with citrate buffer and stored as immobilized cellulase for subsequent use.

2.6 Determination of Substrate Concentration Effect on Cellulase Activity

The assay was based on the ability of the cellulase (enzyme) to hydrolyse empty fruit bunch sample into reducing sugar (glucose). An amount of 0.1 g of steam treated EFB was introduced into test tube containing citrate buffer (pH 4.8) and make up to 10 ml with 2 ml of cellulase. The mixture was placed on water bath maintained at 40℃ and allowed to react for 10 minutes. After the time has elapsed, 3 ml of DNS solution was added to the mixture, to stop the enzymatic reaction. The test tube was covered with laboratory parafilm and placed in a boiling water bath for 10 minutes. After the test tube has cooled, insoluble substances were removed by centrifugation at 3000 rpm for 10 min. Then the absorbance at 540nm against water blank was taken using UV-Vis spectrophotometer. The same procedure was adopted to determine the activity of immobilized cellulase. The sample enzyme activity (U/g) was calculated using equation (1); by substituting the reading of equivalent glucose concentration on the standard curve for the sample and the reaction blank [13].

$$\text{Cellulase Activity (U/g)} = \frac{(C_G - C_B) \times D}{W \times I \times V} \quad (1)$$

where CG is the value from the standard curve for sample enzyme; CB is the value from the standard curve for reagent blank; D is the Dilution factor of the sample; W is the weight of sample taken, g; I is the incubation time, min and V is the volume (ml) of sample solution taken, respectively.

2.6.1 Determination of Optimum Temperature for Activity of Cellulose

Optimum temperature for maximum activities of both free and immobilized cellulase were determined by reacting 1.5 ml of 10 % diluted cellulase-buffer (pH 4.8) with 0.1 g of EFB sample at different temperature (30 to 80°C with 10°C intervals). The contact time was 5 minutes, and DNS method was employed in determining the released reducing sugar using UV/VIS Spectrophotometer.

2.6.2 Determination of Optimum pH for Activity of Cellulase

Optimum temperature for maximum activities of both free and immobilized cellulase were determined by reacting 1.5 ml of 10 % diluted cellulase-buffer of different pH (3-6) with 0.1 g of EFB sample at temperature of 40° C. The contact time was 5 minutes, and DNS method was employed in determining the released reducing sugar using UV/VIS spectrophotometer.

2.6.3 Determination of Substrate Concentration Effect on Cellulose Activity

Different concentrations of substrate ranging from 20 to 100 % (w/w) were prepared and used to assay both free and covalently PEG immobilized cellulase. A volume of 2 ml of 10% diluted cellulase-buffer (pH4.8) was used for the assay at temperature of 40℃ and contact time of 5 minutes. DNS method was employed in determining the released reducing sugar at each concentration by using spectrophotometer.

2.7 Enzymatic Hydrolysis

The EFB sample (1 g) was introduced into 250 ml Erlenmeyer flask. In order to prevent the microbial growth during hydrolysis, 20 mg/ml of sodium azide in distilled water was introduced into the flask. A solution of 2ml of cellulase was mixed with 0.05 M citrate buffer (pH 4.8) and poured into the flask to make a total volume of liquid medium of 30 ml. The Erlenmeyer flask was covered with laboratory parafilm and its contents were heated up in an incubator shaker (CERTOMAT Model, Germany) at 50°C, 160 rpm for 48 hour. Sample was taken for analysis every 8 hours. The reducing sugar released during hydrolysis reaction was quantified by DNS method.

2.8 Enzyme Kinetics

Kinetics refers to the study of the rates at which chemical reactions take place. The rate of an enzyme-catalysed reaction can be expressed in terms of disappearance of the substrate or the formation of the product over time. As the reaction proceeds, both the reverse reaction and forward reaction compete until their rates are equal and at this point equilibrium has been achieved. To account for this kinetic behaviour caused by enzyme-substrate interaction, a model that includes an enzyme-substrate (ES) complex is suitable. The reaction scheme is as shown as in equation (2) [14].

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{K_2} E + P \quad (2)$$

where k_1 and k_{-1} are the forward and reverse rate constants for the formation of the ES complex; and k_2 is the rate constant at which the ES complex converts the bound substrate into product.

Enzymes activate the rates of biochemical reactions by stabilizing the transition state of the reaction, hence lowering the energy barrier that must be exceeded before a reaction can takes place. In enzyme-substrate reaction, The Michaelis-Menten reaction rate is as shown in equation (3).

$$\text{Reaction rate, } v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (3)$$

where v_0 is reaction rate, Vmax is the limiting (maximum) rate attainable, [S] is concentration of substarte (mg/ml), K_m is the Michaelis constant for the reaction.

According to Mazzei *et al.*, (2009)[15], more accurate graphical evaluation of V_max and K_m can be obtained by the Lineweaver-Burk equation:

$$\frac{1}{v_0} = \frac{K_m}{V_{max}}\frac{1}{S} + \frac{1}{V_{max}} \quad (4)$$

2.9 Product Analysis

Analysis was conducted for the purpose of quantification of reducing sugar released during hydrolysis reaction, for free enzyme and immobilized enzyme application respectively. Using DNS method in conjunction with UV-Vis spectrophotometer, the enzymatic hydrolysis efficiency was calculated using equation (5) [16].

$$\text{Hydrolysis efficiency (\%)} = \frac{\text{Released reducing sugar(g)} \times 0.9}{\text{Initial cellulose in substrate(g)}} \times 100 \quad (5)$$

3.0 RESULTS AND DISCUSSION

3.1 Immobilization and Cellulose Activity

The amount of enzyme that was immobilized onto the polyethylene glycol surface was estimated by spectrophotometric enzymatic activity assay, as commonly used for the soluble enzyme. The equivalent reducing sugar released during hydrolysis was substituted in Equation 1 to estimate the cellulytic activity. The activity of free cellulase and PEG immobilized cellulase were calculated to be 1.58 U/mg and 2.38 U/mg respectively. 1.0 U of activity liberates 1µmol of reducing sugars (expressed as glucose equivalents) in one minute. This implies that immobilized cellulase is 50% efficient compare to free cellulase. This is as a result of positive influence of PEG on enzyme desorption from the substrates. As such, the order of enzyme activity enhancement in the presence of PEG corresponds with the increase in cellulose conversion [17].

3.2 Effect of pH on Enzyme Activity

Citrate buffers of different pH, ranging from 3-6 were used to determine the effect of pH on the activity of free and immobilized cellulase respectively at 40°C. It was found that maximum activity of free cellulase was at pH4 and that of immobilized cellulase was at pH4.5 (Figure 1). The increase in pH value could be attributed to the charge nature of interaction between the micro-environment of surface and enzyme due to the coupling with poly ethylene glycol [10]. This result for free cellulase is synonymous to work reported by Zhou (2010)[18], whose value was pH 4, and differs in pH value of immobilized cellulase with 12.5% increase, due to difference in carrier for the immobilization. It shows that immobilized cellulase has better pH stability than the free cellulase.

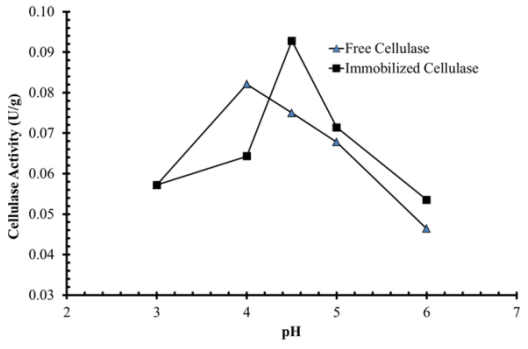

Figure 1 Effect of pH on the activity for free and immobilized cellulose

3.3 Effect of Temperature on Enzyme Activity

Experiments at different temperatures, ranging from 30-80°C at intervals of 10°C were performed to verify the optimum temperature of free and immobilized cellulase respectively at pH 4.8. It was observed that optimum temperature for the free cellulase was 40 °C and that of immobilized cellulase was 50°C (Figure 2). At the respective optimum temperature, free and immobilized cellulase showed maximum activity, and afterward it decreased per 10°C rise in temperature. The increase in activity is basically due to increase in rate constant with temperature and partly due to increase in interfacial area with increasing temperature [19]. The decrease in activity afterward is caused by enzyme deactivation after the respective optimum temperature. This result is almost the same with the similar work published [20], they reported the optimum temperature for free cellulase as 40°C, but 10% differs on the temperature of immobilized cellulase, because of difference in chosen carrier.

As shown in Figure 3, the hydrolysis aided by free cellulase resulted to release of 0.61 g/g substrate at the end of 48-h reaction, while that of hydrolysis reaction activated with immobilized cellulase enhanced the reducing sugar recovery to 0.66 g/g substrate at the end of 48-h. The reducing sugar recovery using free cellulase and immobilized cellulase correspond to the hydrolysis efficiency of 55% and 60% respectively (Figure 3). The enhancement of reducing sugar recovery using immobilized cellulase is as a result of PEG interaction with the lignin on the lignocellulose surface which prevents unproductive enzyme adsorption [21]. As such, the order of enzyme activity was improved and led to efficient cellulose conversion.

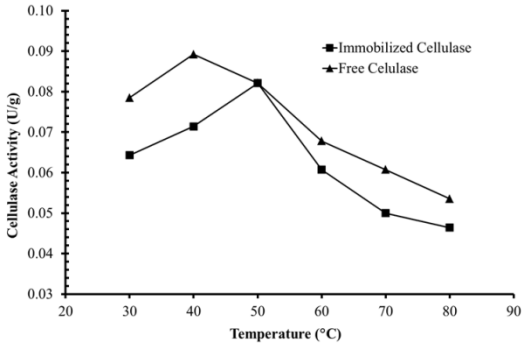

Figure 2 Effect of temperature on the activity of free and immobilized cellulose

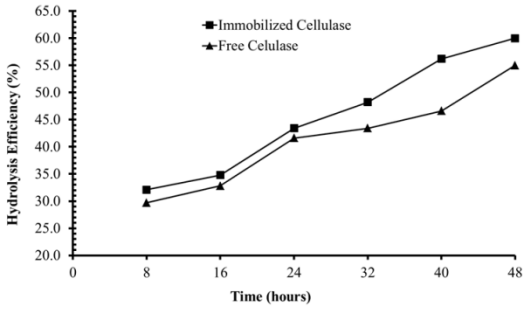

Figure 3 Hydrolysis efficiency versus time for free and immobilized cellulose

3.4 Kinetic Study

The Michaelis-Menten kinetic model of single-substrate reaction was investigated for free and immobilized cellulase respectively.As the enzyme-catalysed reactions are saturated, their rates of catalysis do not show a linear response to increasing substrate. As substrate concentrations increase, the enzyme becomes saturated with substrate and rate reaches Vmax , the enzyme's maximum rate [22]. The apparent kinetic parameters (V_{max} and K_m) of the free and immobilized cellulase were determined by varying concentration of solid cellulose matter (EFB sample) from 20 to 100% in 0.05 mM/L sodium citrate buffer (pH4.8). The varied concentrations of substrates were reacted with free and immobilized cellulase respectively, in order to determine the level of hydrolysis and the reaction velocities. The Michaelis-Menten equation in its original form is not well suited for estimation of the kinetic parameters, Vmax and Km. It is quite difficult to estimate V_{max} accurately from a plot of initial rate (V) and substrate concentration (S) [23].

With the application of Lineweaver-Burk plot (1/V vs1/S) as shown in Figure 4), the Michaelis constant (K_m) and limiting velocity (V_{max}) values for both free cellulase and immobilized cellulase were determined as presented in Table 1. The higher K_m value of immobilized cellulase could be attributed to the PEG interference with the binding of cellulase to extrude substrate, and enable free movement of cellulase to hydrolyze cellulose maximally. The immobilization method can also results in the increase and decrease of these parameters. The parameters are important in characterizing the specificity of an enzyme for a particular substrate, in deciding between steady-state and equilibrium mechanisms and in indicating the role of an enzyme in metabolism. This result conforms in terms of higher value of Km, with the work published previously [19].

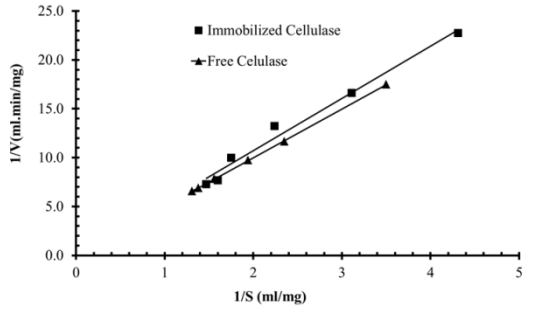

Figure 4 Lineweaver-Burk plot for free and immobilized cellulase

Table 1 Apparent kinetic parameters for free and immobilized cellulose

Parameter	Free cellulase	Immobilized cellulose
Michaelis constant (K_m), mg/ml	171.8	179.2
Limiting velocity (V_{max}), mg/ml.min	34.5	33.5

4.0 CONCLUSION

The cellulase was covalently immobilized on activated and functionalized polyethylene glycol via glutaraldehyde coupling. The immobilized cellulase has demonstrated 50% higher efficiency in terms of activity as compared to free cellulase. It was investigated that factors such as pH, temperature and substrate concentration have significant effects on the activity of immobilized cellulase. Temperature and pH optima of cellulase immobilized on PEG were shifted upward; this would in turn improve the usage of the immobilized cellulase. The kinetic investigation showed that, Km value of immobilized cellulase is higher than that of free cellulase, due to the influence of polyethylene glycol on the binding cellulase to substrate. This results to the enhancement of hydrolysis process and high recovery of reducing sugar. Application of PEG immobilized cellulase could be a better option in an industrial scale of ethanol production.

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