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CELLULASE IMMOBILIZATION USING REVERSIBLE SOLUBLE-INSOLUBLE POLYMER

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ABSTRACT

Immobilization is considered to make the enzyme utilization more cost efficient. Cellulase should be in soluble form to attack the insoluble cellulose as insoluble enzyme is favourable for recycling. Stimuli responsive polymer is a polymer which can change its form reversibly due to stimulus (temperature or pH). N-succinyl chitosan (NSC) is one of smart polymers which is commonly used as a drug carrier, to dress wounds and to make cosmetic materials. In this experiment, NSC was made from 1 gm of chitosan mixed with 0.5 gm of succynil anhydride in 40 mL of dimethylsulfoxide (DMSO) and incubated for 18 – 24 hours at 60oC. The reusability percentage was 87 and it retained its activity at 180 U/mL. Optimum pH for NSC preparation was achieved at pH 4 – 5. A 5 mg EDCCl amount was used for 5 mL mixture to conjugate the NSC with cellulose. It showed its highest activity in terms of reducing sugar production as much as 2.85 mg/mL.

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KEYWORDS

Cellulase; smart polymer; stimuli responsive polymer; N-succinyl chitosan; fermentable sugar

INTRODUCTION

One of the hurdles in enzimatic hydrolysis in the bioethanol process technology is the cellulase cost. In the second generation of bioethanol production, lignocellulosic materials from biomass are considered as the main raw materials. Many efforts have been made for breaking the lignocellulose into lignin and cellulose[1,2]. Cellulase can convert cellulose, the main polymer in biomass, into glucose which can be further fermented to ethanol. Cellulase is currently the third largest industrial enzyme worldwide. It might become the largest volume industrial enzyme, if the cellulosic platform ethanol becomes the major transportation fuel[3]. One way to make enzymatic process economically attractive is to immobilize the enzyme so that catalytic properties are kept and they can be re-used at many times[4].

A robust immobilized enzyme, by definition, has to perform two essential functions, namely the non-catalytic and the catalytic functions. The non-catalytic functions are designed to aid separation, while the catalytic functions are designed to convert the targeting compounds or substrate within a desired time and space[5]. Main problem in enzymatic hydrolysis of cellulose is the diffusion limitation. Cellulase bound on solid matrices and the substrate is not soluble, so cellulase might show poor performance[6]. To meet the requirement of a robust enzyme immobilization method, this work immobilized the cellulase in a stimuli-responsive polymer.

Stimuli-responsive polymers or smart polymers are polymers that respond with dramatic property to changes in their environment such as pH or temperature. This phenomenon is reversible as its system returns to its initial state when the trigger is removed[7]. This polymer can also conjugate with proteins namely responsive polymer-protein bioconjugates (RPPBs), which are synthesized by different covalent and non-covalent approach in distinct physical forms[8]. Having those kinds of properties made them possible to act as an enzyme immobilizing support, N-succinyl-chitosan (NSC) is one polymer that shows soluble-insoluble characteristics with pH change. The effectiveness of employing cellulase immobilized on NSC (NSCC) for extracting flavonoids from Ginkgo biloba leaf powder has been investigated by Zhou[6]. This study produces fermentable sugar by using N-succinyl-chitosan as cellulase immobilization support.

MATERIALS AND METHODS

(i) Materials

Novoprime B-959 (Novozyme, China) with average activity 151 FPU/mL. Succinic anhydride 99+% (Sigma-Aldrich, India), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Sigma-Aldrich, USA) or EDCCl, Dimethyl sulfoxide 99% (Junsei chemical Co., Ltd., Japan), Chitosan (2-Amino-2-deoxy-(1-4)-β-D-glucopyranan) (Fluka, Switzerland). Buffer solution contain: Citric acid 99.5%, MW:210.14 (Shimakyu, Japan), Sodium citrate 99%, MW:294.10 (Shinyo, Japan). DNS reagents: 3,5-Dinitrosalicylic acid, 98+% (Lancaster, England), Potassium Sodium Tartrate or Rochelle Salt 99%, FW: 282.22 (Duksan, Korea), Sodium sulfite 95%, FW:126.04 (Junsei Chemical, Japan),

Sodium hydroxide 96+%, FW:40 (Yakuri, Japan), Phenol 98%, FW:94.11 ((w/v) [9].

(ii) Immobilization of Cellulase (NSCC)

A 2 mL of the NSC's were mixed with 3 mL citrate buffer pH 3 – 8 and also with N-(3- Yakuri, Japan). D-(+)-Glucose, minimum 99% GC (Sigma, USA), Carboxymethylcellulose (CMC).

(iii) Preparation of N-Succinyl Chitosan (NSC)

Certain amount of chitosan powder: 0.5 g; 1 g and 2 g, were mixed with dimethyl sulfoxide (DMSO) 40 mL. Succinic anhydride was added into the solution progressively as much as 0.5 gr prior to shaking at 130 rpm for 18 hours in temperature ranging from 50 – 60oC. After incubation, the solution was separated using vacuum filter. The filtrate was then dispersed in 50 mL of distilled water and adjusted to pH 11 – 12 using NaOH 5% Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCCl) as much as 5 and 10 mg. This solution was stirred for 30 minutes. Then 1 mL cellulase was added to the mixture. After having stirred well, the NSCC was then stored at 4oC.

(iv) Analytical methods

Filter mesh with 500 micrometer size was used in a simple filtration system for getting the NSC precipitate after the pH adjusted to 1 for two times. Reusability percentage is defined as

$$\text{Reusability} = \frac{\text{amount of NSC from 2nd filtration}}{\text{amount of NSC from 1st filtration}} \times 100\%. \quad (1)$$

The activity assays were carried out over the pH range 3 – 8 and temperature 45oC to determine the pH and the temperature profiles for the free and immobilized cellulase. The results of pH and temperature were presented in a normalized form, with the highest value of each set being assigned as the value of 100% activity. To measure glucose concentration, the sample solution was diluted by the dinitrosalicylic acid method[10,11]. Sample absorbance was measured by UV Spectrophotometer HACH, US/DR-4,000U at 575 nm.

The activity of cellulase was measured by using 1% (w/v) CMC as the substrate and acetate buffer (pH 4) as the medium. Free cellulase (1 mL) was added to 4 mL of the CMC solution and incubated at 45oC for 25 min. The amount of generated glucose was determined by the 3,5-dinitrosalicylic acid reagent (DNS) method. One unit of activity was defined as 1 micromol of generated glucose/min.

The immobilized cellulase solution was added to the same assay medium and incubated at 45oC for 25 min. Then the pH of the solution was adjusted to 3 by HCl. The resulting mixture was filtered, and the filtrate was collected to determine the amount of generated glucose.

The retained activity of immobilized cellulase, determined by the percentage of the activity of immobilized cellulase in the activity of free cellulase used for binding, was calculated according to the equation:

Retained activity (%) = UIC/Utotal x 100 (2)

Where UIC is the activity of immobilized cellulase and Utotal is the activity of free cellulase used for binding.

RESULTS AND DISCUSSIONS

1. Ratio of Succinyc Anhydride and Chitosan to N-Succinyl Chitosan Formation.

Succinyl chitosan was obtained by introduction of succinyl groups into chitosan N-terminal of the glucosamine units as shown in Figure 1[8].

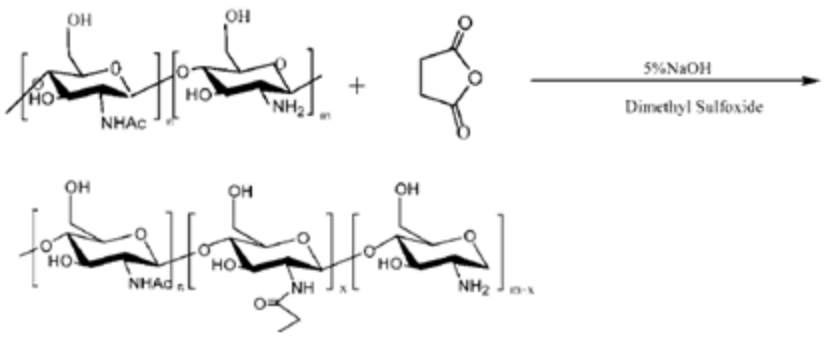

Figure 1
Synthesis Route of N-Succinyl-chitosan [8]

The ability of immobilized carrier to be reused is the main purpose for this step. The solution of NSC after 24 hour incubation between succinyc anhydride and chitosan was highly viscous (Figure.1 a). By adjusting the pH, the solution changed its phase into an insoluble form as seen in figure 1. This precipitate, pointed by red arrow, then was filtered using a simple filtration system for twice to find out the reusability percentage of the NSC. In all ratio, the NSC can be precipitated by adjusting the pH to 1 but the lowest chitosan amount shows the smallest precipitated area and the weak structure. This might happen due to excess solvent volume in the smallest chitosan amount. Hence, the ratio between chitosan, succinyc anhydride and the DMSO were not suitable for the NSC formation [6].

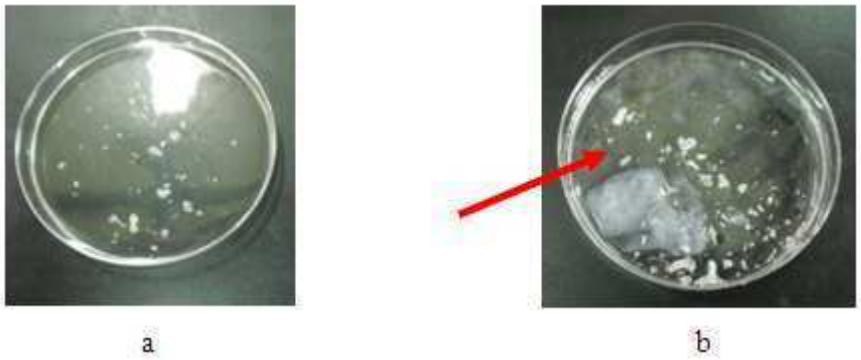

Figure 2.
Pictures of N-Succinyl chitosan in different pH (a) at pH >4, (b) pH<4

The reusability percentage of NSC is listed in Table 1. The highest percentage was achieved by using ratio 1:4 of succinyc anhydride to chitosan amount with more than 90% of reusability. This means that the chitosan amount, which was available in the solution, was succinylated perfectly by the succinyc anhydride. Since 1:4 is the smallest ratio of succynic anhydride and chitosan, which was used in this work, there is a possibility that by using smaller ratio the reusability percentage might show higher value.

Table 1
Effect of succynic anhydride to chitosan to phase change

Ratio	% of reusability
1:1	21%
1:2	87%
1:4	93%

3.1Effect of pH in immobilization procedure on the activity of immobilized cellulase

In the immobilization procedure, EDCCl first reacts with the carboxyl groups on NSC, forming a reactive and unstable amine-reactive O-acylisourea intermediate. This intermediate then reacts with the amine groups of enzyme, yielding stable amide bonds between enzyme and NSC. The reaction was dependent on the pH value of the solution, so the effect of pH in immobilization procedure on enzyme activity was important to investigate. Immobilization was carried out in buffer solution from pH 3.0 to pH 8.0. As shown in Figure 2, the maximum activity of immobilized cellulase reached 178 units/mL at pH 4.0. When pH was 3.0, NSC was insoluble and it was not active by EDCCl, so the activity of immobilized cellulase was unavailable. When pH was above 4.0, NSC was soluble and the solution was homogeneous. At pH 4.0 to 5.0, EDCCl was in the most reactive state to react with carboxyl groups on the support, so the NSCC could show high activity in pH 4 - 5. The significant decrease of NSCC activity above 5.0 was due to the instability of free cellulase against the increase of pH. Zhou had also shown similar results but by employing EDC as condensing reagent and coupling trypsin on poly(methylmethacrylateethyl acrylate-acrylic acid) latex particles, it was found that a maximum value of enzyme activity could be achieved at pH 5.0[6].

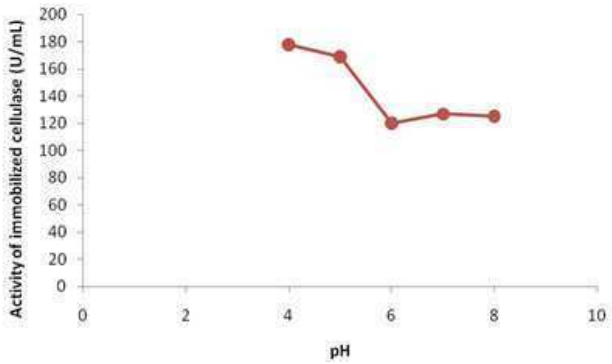

Figure 3
Effect of pH in immobilization procedure on the activity of immobilized cellulase at 60^{o}C incubation

3.2Effect of chitosan amount to reducing sugar production

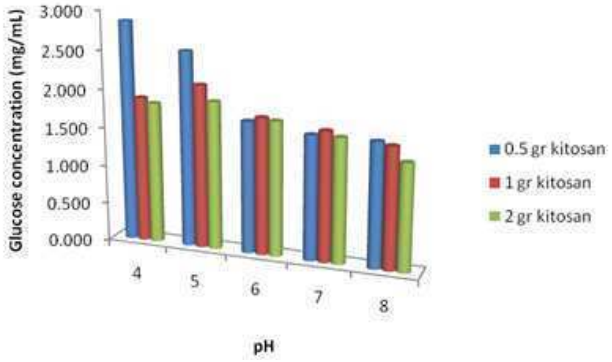

Figure 4
Effect of chitosan amount in reducing sugar production

Chitosan amount in the NSC formation has made the bond stronger between succinyc anhydride and chitosan. But apparently it reduces the activity of immobilized cellulase. The higher chitosan amount made the structure of NSC become more rigid, which, unfortunately, make this carrier rigidity limiting to the enzyme activity[8]. The optimum chitosan amount in this experiment is 1 gr of chitosan for 0.5 gr succinyc anhydride. Increasing the chitosan amount has a good result for the non-catalytic performance (recycle-ability) but it also has a contrary effect on the catalytic performance.

3.3Effect of EDCCl amount to reducing sugar production

EDCCl is one example of compounds containing the carbodiimide functionality. It can be used as dehydration agents and are often used to activate carboxylic acids towards amide or ester formation. Carbodiimides, commonly obtained as the hydrochloride, is a water soluble carbodiimide which is typically employed in the 4.00 – 6.00 pH range. It can be used as a chemical crosslinker for functional polymers, reacting with the carboxylic acid groups of the polymers which then can bond the amino group in the reaction mixture.

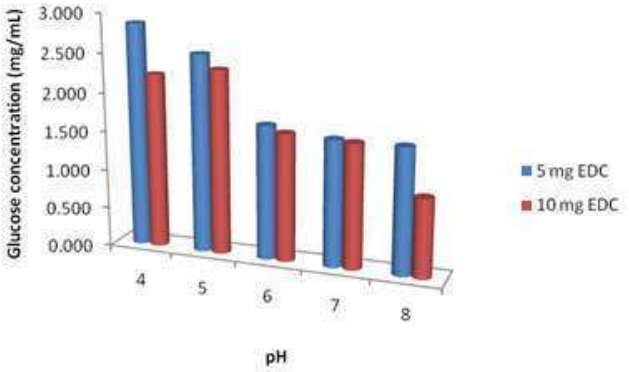

Figure 5
Effect of EDCCl amount in reducing sugar production

In this work EDCCl is used as a condensing reagent to couple carboxyl groups on NSC to the amino groups of lysine residues in the protein[6]. The optimum amount of EDCCl to produce highest reducing sugar in present work is 5 mg per 5 mL of NSC and buffer mixture which can be seen in figure 4. The increase in the amount of EDC resulted in more activated carboxyl groups and higher immobilized enzyme activity. However, when an excessive amount of EDC was introduced, the amide bonds could be formed not only between cellulase and support but also between enzyme molecules. This partial cross-linking of enzyme restricted the conformation mobility of the molecules and thus led to the loss in enzyme activity. Similar observations had already been reported by other researchers[6]. In this study, the optimum EDCCl amount for cellulase immobilization was 5 mg.

CONCLUSION

N-Succinyl Chitosan was successfully prepared in this experiment. The optimum amount of succinyc anhydride was 0.5 gr, chitosan 1 gr and DMSO 40 mL in 60oC for 18-24 hours incubation. The above complies to the requirement of a robust enzyme, namely the non-catalytic function with 87% reusability and catalytic function almost 180 U/mL retain activity. A 5 mg EDCCl amount used for 5 mL mixture to conjugate the NSC with cellulase showed highest activity in terms of reducing sugar production, as much as 2.85 mg/mL. There should be a further investigation for re-cycling the immobilized cellulase and also to apply it in the ethanol production.

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