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Immobilization of Cellulase on Non-Porous Ultrafine Silica Particles

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The immobilization of cellulase onto non-porous ultrafine silica particles was studied. Cellulase was extracted from a Trichoderma reesei culture after partial purification with ammonium sulfate (pH = 5.0), which was then immobilized onto non-porous ultrafine silica particles, with or without the use of glutaraldehyde as a crosslinking agent. Cellulase was immobilized by adsorption onto ultrafine silica particles efficiently, as well as by covalent cross-linking with glutaraldehysde. Increasing the concentration of the free form of enzyme increased the amount of immobilized cellulase. The maximum enzyme immobilization happened at the free enzyme concentration of 0.48 mg/ml. In general, the optimum pH for immobilization was found to be 5.0, which is close to the pI of the enzyme. The relative activity of the immobilized enzyme was also increased as the amount of immobilized enzyme was increased. However, immobilization of the enzyme on the ultrafine silica particles, with or without the use of glutaraldehyde, showed almost the same enzyme activities. The immobilized cellulase showed a higher thermal stability, with respect to temperature, compared to the free cellulase

INTRODUCTION

Cellulosic material is an abundant renewable source that can serve as a substrate for the production of chemicals and fuel ethanol by chemical or enzymatic conversion [1]. Cellulase catalyses the hydrolysis of the β-1,4 glycosidic bonds of the crystalline complex of cellulosic molecules to produce glucose. As mentioned before, this conversion can be done either by chemical or enzymatic hydrolysis. However, the economic, environmental and operational advantages of the enzymatic process over the chemical route have now been realized and enzymatic hydrolysis has attracted significant attention. On the other hand, for the industrial application of cellulase, its immobilization, which allows the condition of repeated use of the enzyme alongside retaining its activity, has been recently investigated [2-5].

Cellulase has been immobilized by several physical and chemical methods, such as, cross linking [2], copolymerization [3], fiber ultra-filtration [4,5], aqueous 2 phase systems [6,7] and modification of cellulase [8]. In most cases, the carriers used were mainly insoluble organic materials, like copolymers, natural polymeric derivatives and fibers. The problem associated with using organic carriers is their limited capacity for reuse of the immobilization matrix, which, therefore, creates an organic materials disposal problem. On the other hand, the inorganic carriers are more expensive than organic ones; but, they have an advantage over the organic carriers as being reusable, which, in some circumstances, can decrease the cost of immobilization support [9].

In the present work, the non-porous silica particles were used for the first time as enzyme carriers for the cellulase immobilization. The element of novelty in this paper is the use of this particular type of silica as a carrier for the cellulase enzyme. In previous works, the immobilization of enzymes, such as α-amylase and papain, onto ultrafine silica particles, was studied [10]. This work presents the adsorption of partially purified cellulase on ultrafine silica particles, with and without the use of glutaraldehyde, a covalent cross-linking agent.

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MATERIALS AND METHODS

Microorganism, Chemicals and Culture Conditions

The ultrafine silica particles (USP Snowtex 30) were kindly gifted by Kobe University (Kobe, Japan). USP Snowtex 30 (SiO_2: 30.4 wt%) is a colloidal silica solution made by dispersing negatively charged, amorphous silica particles in water. OH ions exist on the surface of the particles with an electric double layer formed by alkali ions. The particles are spherical in shape and the suspension is clear to opalescent, with a viscosity and density of 10 mPa.s and 2.15 g/ml at 25°C, respectively. The average diameter of these ultrafine particles was measured by the Dynamic Light Scattering method and found to be 14.8 ± 0.2 nm. The surface area of the silica particles is 25 m^2/g. Other chemicals were of analytical grade and purchased from Merck AG, Fluka AG (Switzerland) and Sigma Chemical Ltd. (USA). Trichoderma reesei PTCC 5142 was grown in a culture medium consisting of the following components: Microcrystalline cellulose 1%(w/v), $(NH_4)_2SO_4$ 1.4%(w/v), KH_2PO_4 2.0%(w/v), Urea 0.3%(w/v), $CaCl_2$ 0.4%(w/v), $MgSO_4.7H_2O$ 0.3%(w/v), Peptone 1.0%(w/v), $FeSO_4.7H_2O$ 0.005%(w/v), $MnSO_4.H_2O$ 0.0016%(w/v), $ZnSO_4.7H_2O$ 0.0014%(w/v) and $CoCl_2$ 0.0037%(w/v). Aliquots (100 mL) of the culture medium were dispensed into 500 mL conical flasks with lint caps and sterilized at 121°C for 20 min. The flasks were inoculated with T. reesei and incubated on a rotary shaker (200 rpm) at 28°C for 12 days. The samples of the microbial culture were first centrifuged (12000*g for 30 min, at 4°C). The culture filtrate was collected and the enzyme solution was then partially precipitated and purified using ammonium sulfate salt (pH = 5). The precipitated fraction of the enzyme between 30% and 70% of the ammonium sulfate saturation was collected by centrifugation (10000*g, 20 min, 4°C), then, dissolved in a 0.05 M sodium acetate buffer (pH = 4.8) and dialyzed against the same buffer.

Enzyme Immobilization Procedures

Two kinds of enzyme immobilization were studied: The physical adsorption of the enzyme on the surface of non-porous ultrafine particles and with the use of glutaraldehyde as a covalent cross-linking agent. An enzyme solution (4 mL) at the desired concentration was mixed with an equal volume of the suspension of ultrafine silica particles and also in the presence of 0.1 ml of 1% glutaraldehyde solution. The immobilization was carried out in a shaker for 2 h at 30°C. The ultrafine silica particles were then recovered by centrifugation (10000*g, 30 min, 4°C) and resuspended in the 0.05 M sodium acetate buffer (pH = 4.8). The immobilized enzyme molecules were also washed and resuspended in the 0.05 M sodium acetate buffer (pH = 4.8). In both cases, the amount of immobilized enzyme was calculated from the differences between the initial and equilibrated protein concentration.

Analyses (Enzyme Activity and Protein Content)

The enzyme activity was measured by the carboxymethylcellulase activity method. This method is based on the measurement of the amount of glucose produced during the hydrolysis of CMC (carboxymethylcellulose) solution [11]. A 1%(w/v) CMC solution in 0.05 M acetate buffer (pH = 4.8) was used as the substrate. Then, 2 ml of this substrate solution was pre-incubated at 50°C and, after a few minutes, 2 ml of the enzymatic solution was added. The reaction was stopped after 10 minutes by putting the enzyme solution tubes in a boiling water bath for a couple of minutes. Finally, the amount of reducing sugar (glucose) was measured using the DNS method and a glucose solution was used as the standard [10]. One unit of enzyme activity is defined as the amount of enzyme producing 1 μmole of glucose per minute at 50°C and pH = 4.8 from a 1%(w/v) aqueous solution of CMC.

$$\text{Activity of cellulase } (\mu\text{mol/ml.min}) = (1000\ w)/(MVt),$$

where, w is the amount of produced glucose, M is the molecular weight of the glucose, ν is the volume of the measured sample and t is the reaction time, respectively. The relative activity of immobilized cellulase is the ratio of the specific activity of the immobilized enzyme to that of the free enzyme under the same conditions. The activities measured and plotted in the figures are the result of the average of the triple measurement.

$$\text{Relative activity} = \frac{(\text{specific activity of immobilized cellulase}) \times 100\%}{(\text{specific activity of free cellulase})}.$$

The protein concentration was measured by the Lowry method using bovine serum albumin as the standard [11].

Thermal Stability of the Immobilized Enzyme

The thermal stability of the immobilized enzyme versus free enzyme was measured as follows: A solution of 0.55 (mg/m^2) immobilized cellulase was taken, along with a 1%(w/v) CMC solution in 0.05 M acetate buffer (pH = 4.8). Then, the enzyme sample was incubated at different temperatures ranging from 30°C to 70°C. The equivalent amount of free enzyme that has the

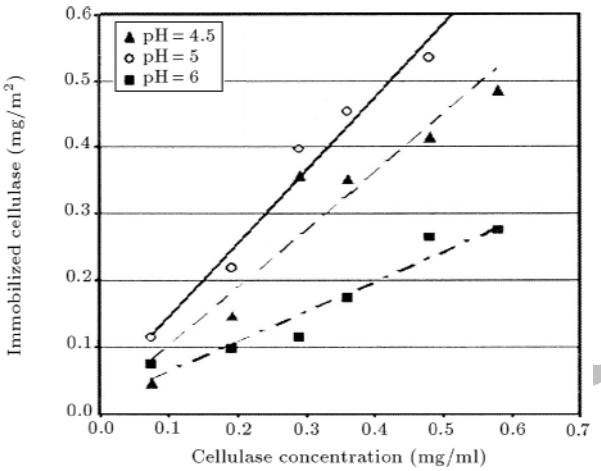
same enzyme activity (0.4 mol/ml.min) as 0.55 mg/m^2 of immobilized cellulase at room temperature, was estimated. Then, the free enzyme samples were placed under the same conditions as the immobilized cellulase in the water bath. The reaction was stopped after 10 minutes by putting the enzyme solution tubes in the boiling water bath for few minutes. Finally, the activities of the free and immobilized cellulase at different temperatures were measured and compared.

RESULTS AND DISCUSSION

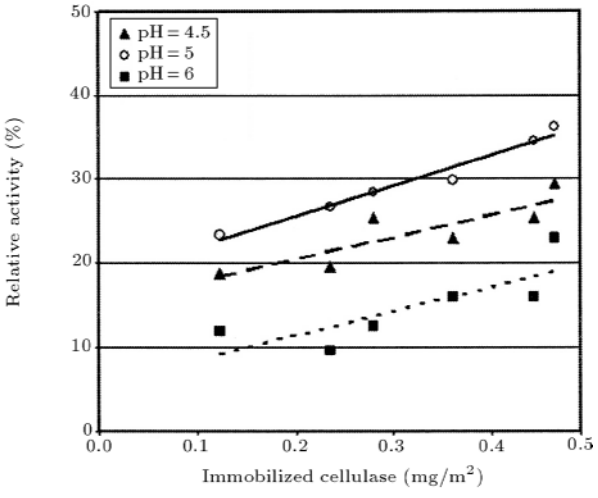
Enzyme Immobilization

Figure 1a shows the amount of adsorbed cellulase on ultrafine silica particles at various pH values. Figure 1b shows the amount of adsorbed enzyme on ultrafine silica particles in the presence of glutaraldehyde as a covalent cross-linking reagent. In these graphs, the amount of immobilized enzyme, which is defined as the weight of immobilized protein per unit of surface area, is plotted against the residual protein concentration. In both cases, the initial slopes and plateau values were affected by pH. The plateau values exhibited a maximum near pH = 5.0, which is close to the isoelectric pH of this enzyme, and decreased significantly when pH was changed above or below the pI value. This result is consistent with the previous reports [9]. Probably, a reduction in the electrostatic repulsion between protein molecules near the isoelectric pH yields a higher packing density on the particle surface. On the other hand, an increase in the electrostatic repulsion between protein molecules and negatively charged surfaces reduces the enzyme immobilization amount at pH values above the isoelectric pH. Figure 2 shows that the amounts of immobilized enzyme on the silica particles, with and without use of the covalent cross-linking agent, were almost identical at pH = 5. The amount of immobilized enzyme with covalent cross-linking agent increased with an increase in the concentration of the enzymatic solution and reached 0.5 mg/m^2 at 0.58 mg/ml of the initial enzyme concentration.

Figures 3a and 3b present the relationship between the amount of immobilized enzyme (with or without use of glutaraldehyde) on the relative activity of the immobilized enzyme at pH=5. Both plots showed that the relative activities increased with an increase in the amount of immobilized enzyme. At 0.45 mg/ml of the immobilized enzyme concentration, the relative activity of the immobilized cellulase reached 30% of the free enzyme activity.



(a) Without covalent cross-linking reagent at various pHs



(b) With covalent cross-linking reagent at various pHs

Figure 1. Amount of cellulase adsorbed on ultrafine silica particles.

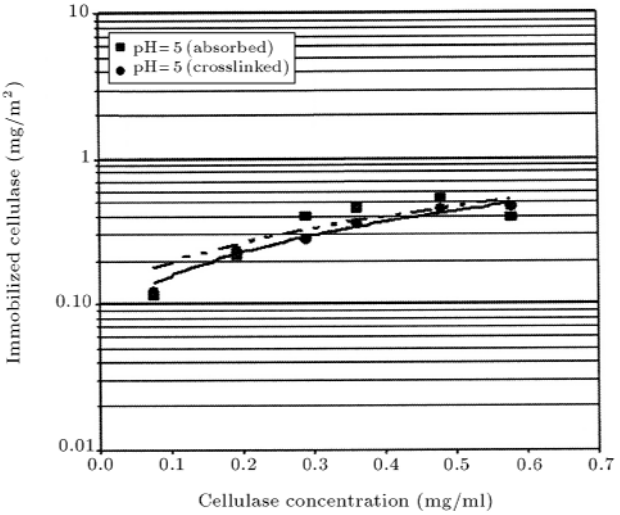


Figure 2. Comparing the amount of adsorbed enzyme on ultrafine silica particles with and without covalent cross-linking agent.

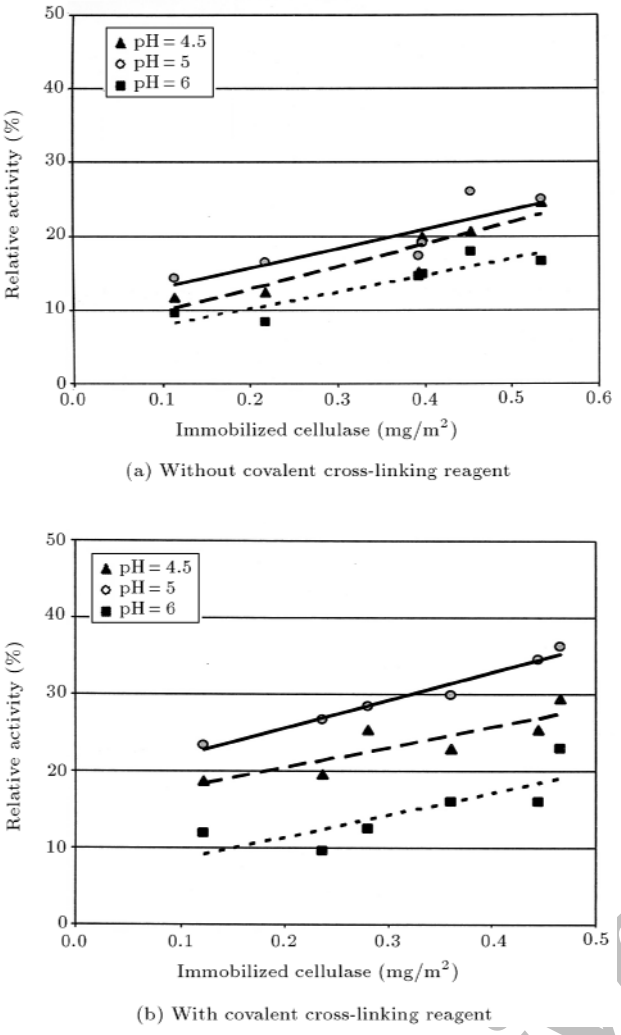


Figure 3. Relative activities of cellulase adsorbed on ultrafine silica particles.

Thermal Stability of the Immobilized Enzyme

Figure 4 shows the stability of the free and immobilized cellulase as a function of temperature. Obviously, the activity of the immobilized cellulase varied in a wider range under the effect of a raise in temperature, compared to the activity of the free enzyme. As temperature is increased, the viscosity of the CMC solution was decreased and the mobility of the CMC molecules increased considerably. All these events will help the access of the substrate to the immobilized enzyme and will provide a proper atmosphere for reaction between the enzyme and substrate. Thus, the activity of the immobilized enzyme will increase to a higher value, whereas it will be lower at lower temperatures.

CONCLUSIONS

In general, immobilization constrains the flexibility of an enzyme, thus, lowering the specific activity of the

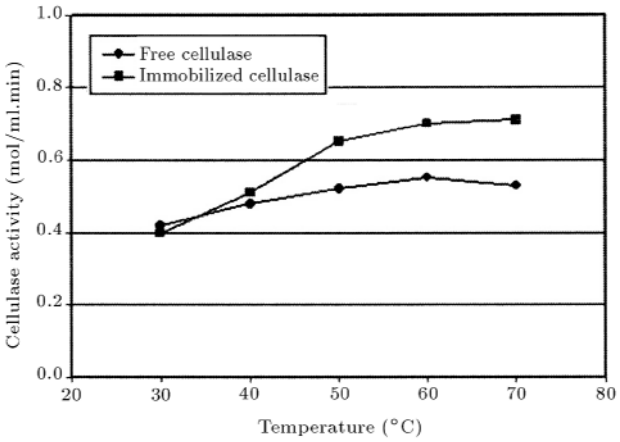


Figure 4. Thermal stability of adsorbed enzyme on ultrafine silica particles versus temperature.

initial enzyme state when compared with the unmodified enzyme. This constrained atmosphere should also hinder the unfolding of the enzyme, thus stabilizing the enzyme. Two kinds of immobilized enzyme were studied in this work: Physical adsorption and covalent cross linking with the use of glutaraldehyde. In both cases, the amount of adsorption was high at a pH around the pI of cellulase, which decreased by either an increase or decrease of pH from the pI value. Meanwhile, a higher enzyme activity was observed at the higher amount of adsorbed enzyme. The lower amount of adsorbed enzyme at higher pH can be attributed to the electrostatic repulsion between enzyme molecules and negatively charged particle surfaces. On the other hand, a higher activity at a higher amount of adsorbed enzyme can be attributed to less conformational changes upon enzyme adsorption, because the interaction between particle surfaces and enzyme molecules is weakened by a reduction in the area available for adsorption and an increase in the repulsive force between adsorbed enzyme molecules as the amount of adsorption increases. Covalent cross-linked cellulase with glutaraldehyde also retains good activity (maximum at 3% glutaraldehyde concentration).

The thermal stability of immobilized cellulase onto silica particles was improved as compared to the free enzyme. As one of the operational difficulties of the hydrolysis of the cellulosic materials by cellulase is the high operational temperature, the immobilization of cellulase will increase the efficiency of the hydrolysis process. Therefore, these particles, lacking internal mass transfer limitations and having good thermal stability, can be suitable candidates for industrial application.

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