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Immobilization of Cellulase Using Polysulfone Membrane

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Cellulase has been successfully immobilized in polysulfone by means of cross-linking and filtration with pressure The optimum conditions for the preparation of the immobilized enzyme and the characteristics of the immobilized enzyme were obtained. The cellulase exhibited improved stability in reaction conditions over a broad temperature range and an optimal pH value of 3.6 after being immobilized on the polysulphone membrane. The value of the Michaelis constant (K_m) of the immobilized cellulase (26.853 g/L) was higher than that of the free one (11.577 g/L), whereas the maximum velocity (V_{max}) was lower for the free one. Storage stability and temperature endurance of the immobilized cellulase were found to increase greatly and the immobilized cellulase retained 38.5 % of its initial activity after 6 successive batch reactions.

Key Words: Cellulase, Immobilization, Filtration, Polysulphone membrane.

INTRODUCTION

Cellulosic material is an abundant renewable resource that can serve as substrate for the production of chemicals and fuel ethanol by enzymatic conversion[1,2]. Cellulases are commonly used in various industries, including the food, agriculture, textile, animal feed, pulp and paper,as well as in research development[3,4]. But cellulase are costly and easy to inactivate in free form[5], therefore immobilization is considered a promising way to elongate their lifetime and improve their stabilities. Membrane-immobilized enzymes may serve as model systems for enzymes for less expensive, more stable and reusable alternatives to free enzymes[6]. In addition, the membrane bioreactor is easy to scale-up[7].

Different from covalent binding immobilization[8,9] and physical adsorption immobilization[10,11], cross-linked enzyme crystals or aggregates[12-14] have attracted interest as a carrier-free alternative to conventional immobilized enzymes. In comparison to other methods, there is no covalent binding between the support material and enzymes by using inert polymers such as polysulfone[15]. This makes it possible to regenerate the support materials by removing the enzyme gel through backwash with a high-pressure water or gas[16].

This paper reports the immobilization of cellulase onto a microporous polysulfone which was cross-linked with glutaraldehyde before filtration.

EXPERIMENTAL

Cellulase was purchased from Hua Shuo Fine company, glutaraldehyde (GA) 25 % and the substrate carboxymethyl cellulose (CMC) were obtained from Xilong chemical company (Shantou). Other solvents and chemicals were purchased from Kaifeng Chemical Reagents Company. Flat polysulfone membranes with cut-off molecular weight of 10000 Da were purchased from Ande Membrane Separation Technology & Engineering(Beijing).

Immobilization of cellulase: Cellulase solutions were prepared by adding cellulase powder to acetate buffer solution (0.2 mol/L, pH 4.6) and glutaraldehyde (25 %). Then the reaction mixture was shaken for 0.5 h at 4 ºC. The polysulfone flat membrane was put into a filtration cell and the cross-linked cellulase solution was filtrated for 2 h under 0.5 MPa by compressed nitrogen. After that the immobilized membrane was rinsed by distilled water.

Determination of proteins: The original enzyme protein concentration and the concentration after filtration were measured by the Bradford method with UV-vis spectrophotometry[17]. The amount of cellulase immobilized on membrane =

$$\frac{c_1\nu_1 - c_2\nu_2}{A}$$

where c_1 = initial concentration of cellulase (mg/mL), ν_1 = initial volume of filtration solution (mL), c_2 = concentration of cellulase afer filtration and rinse (mg/mL), ν_2 = volume afer filtration and rinse(mL), A = area of membrane respectively (cm^2).

Measurement of cellulase activities: A solution of CMC was chosen as the substrate. 0.2 mol/L acetic acid buffer (pH 4.6) was used as the medium. Then 4 mL substrate in a beaker was placed in a 40 ºC water bath for 5 min by no stirring so that CMC can be hydrolyzed. Then measure the amount of glucose production from CMC with a spectrophotometer according to Ghose[18]. Activity of cellulase (μmol/min cm^2) = w/(A·t), where w is the amount of produced glucose, A the area of membrane and t is the reaction time, respectively.

RESULTS AND DISCUSSION

Influence of parameters on the activity and the amount of immobilized cellulase

Concentration of cellulase: The concentration of cellulase has large effects on the density of enzyme loaded on the membrane and the performance of biocatalytic membrane. In this study, different amount of enzyme were immobilized on the membrane by varying the concentration of enzyme solution.

As shown in Figs. 1 and 2, the increase of the concentration of enzyme led to the great improvement of the loading density and apparent reaction rate, further increase in concentration caused a thick gel layer to form on the membrane surface and increased the mass transfer resistance of CMC to cellulase. Simultaneously, the glucose produced by hydrolysis could not be removed fast enough, which would inhibit the enzymatic reaction.

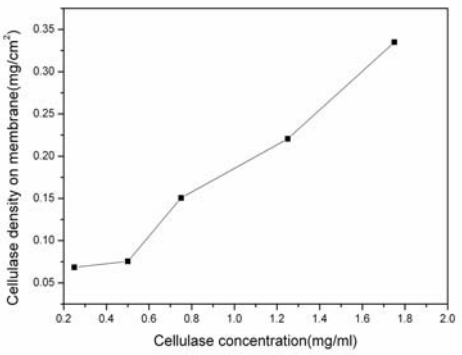

Fig. 1. Effect of cellulase concentration on loading density of membrane

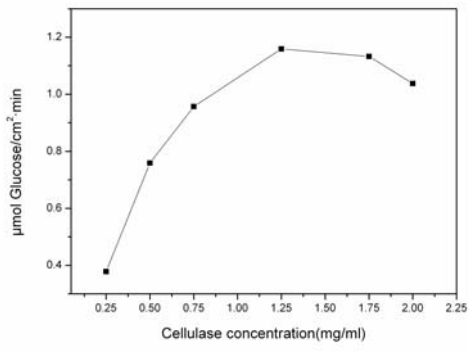

Fig. 2. Effect of cellulase concentration on hydrolysis rate

Concentration of glutaraldehyde: Figs. 3 and 4 indicate the highest apparent reaction rate achieved with7 % glutaraldehyde, which was the optimal concentration of the cross-linking agent. Too low glutaraldehyde concentration made gels not stable enough to be held on the membrane surface, the immobilized enzymes would be removed when the immobilized membrane was used. Too high glutaraldehyde concentration would damage the activity sites of cellulase.

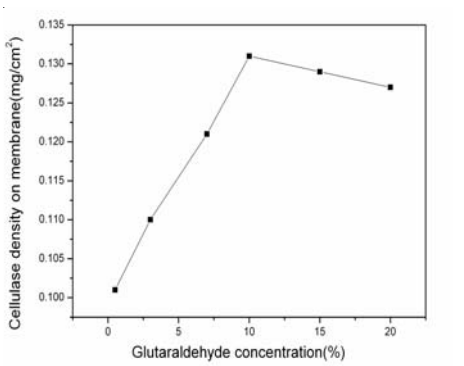

Fig. 3. Effect of glutaraldehyde concentration on loading density of membrane

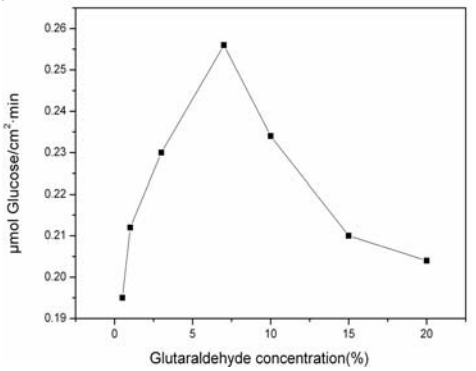

Fig. 4. Effect of glutaraldehyde concentration on hydrolysis rate

Cross-linking time: It has been well demonstrated that crosslinking reaction has a great impact on the activity of immobilized enzymes because crosslinking could destroy the active site of enzyme[19]. The effect of crosslinking by glutaraldehyde on the activity of immobilized cellulase was determined by changing crosslinking time and the results are shown in Fig. 5.

Results reveal that with increasing the crosslinking time, catalytic effciency of immobilized cellulase was firstly increased and then reduced gradually.The optium time is 120 min in this condition.

Filtration time: Filtration has mainly impact on cellulase density on membrane. In this study, the amount of loading cellulase was measured by increasing the filtration time to seek for the optimum condition. As Fig. 6 shown, the optimum filtration time is 120 min in this study.

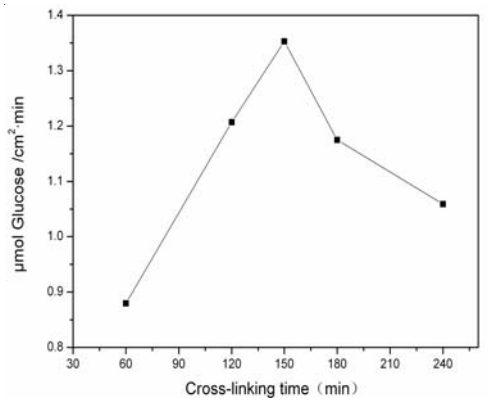

Fig. 5. Effect of cross-linking time on hydrolysis rate

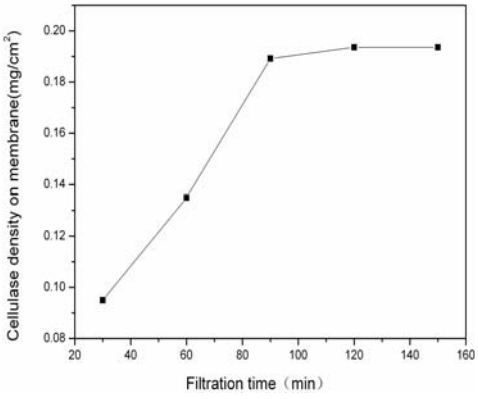

Fig. 6. Effect of filtration time on loading density of membrane

Stability of immobilized cellulase

pH activity: The pH activity of immobilized and free enzymes were determined by measuring the rate of hydrolysis of carboxymethyl cellulose (CMC) in acetate buffer, pH 3.6-7.6 and phosphate-citrate buffer pH 2.6.Free and immobilized enzyme solutions were reacted with CMC for 0.5 h at 40 °C. Activity retention of both enzymes at various pH values are shown in Fig. 7.

The optimum pH of the immobilized enzyme and free enzyme were 3.62. Compare with free cellulase immobilized cellulase has better pH stability. The similarity in pH-activity patterns of free and immobilized enzymes is probably due to the nature of polysulfone membrane.

Temperature activity: Free and immobilized enzymes were reacted with CMC in 0.2 mol/L acetate buffer (pH 4.6) at different temperatures between 30 and 80 °C. The retentive activity of each hydrolysate was assayed after 0.5 h reaction. The results are presented in Fig. 8. The optimum temperature of both the free and the immobilized enzyme was 50 and 60 °C. Compared with the free enzyme, immobilized cellulase has wider temperature range.

Recycling stability: The recycling stability of immobilized cellulase was examined by measuring the activity repeatedly. As seen from Fig. 9, the relative activity of the immobilized cellulase compared to its initial value in percentage decreased along with the reusing times and the immobilized enzyme retained over 30 % of its initial activity after six cycles of reuse. Such reusability is advantageous for the continuous use of this enzyme in industrial application[19]. The loss of catalytic

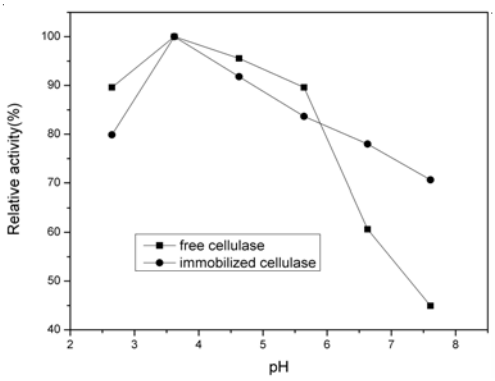

Fig. 7. Activity of immobilized cellulase and free cellulase with respect to pH, CMC concentration 0.62 % (w/v), tempareture 40 ºC and time = 0.5 h

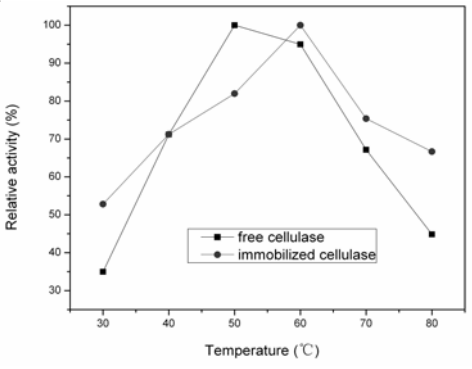

Fig. 8. Activity of immobilized cellulase and free cellulase with respect to temperature. CMC concentration 0.62 % (w/v), pH 4.6 and time = 0.5 h

activity may be explained by the following reasons. Firstly, the adsorption cellulase lost during the process of measurement. Second, the production has toxic to the cellulase.

Kinetic parameters: At the given condition, the beginning velocities of CMC hydrolysis with a series of CMC concentrations were determined by measuring the amount of glucose production. According Lineweaver-Burk method[20] plots of 1/V *versus* 1/[S] with free cellulase and immobilized cellulase were linear as seen in Fig. 10.

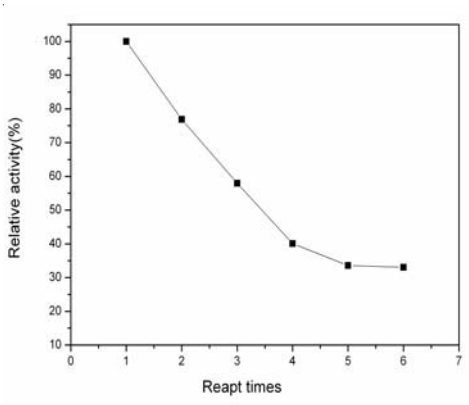

Fig. 9. Relative activity of immobilized cellulase after repeated assay

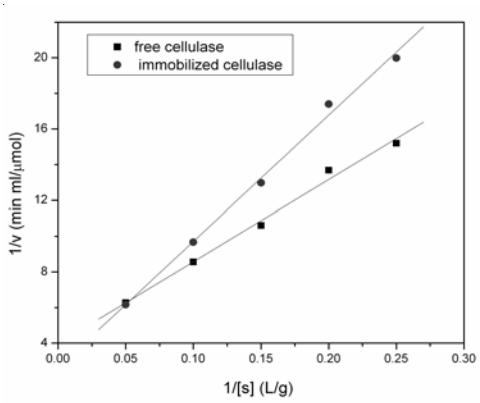

Fig. 10. Relationship of the velocity of CMC hydrolysis and CMC concentration, pH = 4.6, 0.2 mol/L acetate buffer, temperture = 40 ºC and time = 0.5 h

The values of K_m and V_m can be calculated from slopes and intercepts of the two lines as shown in Table-1. It has no coincidence conclusion about the kinetic parameters of cellulase. Xiao-yan Yuan[21] results indicated that the K_m of immobilized cellulase is more higher than free higher than that of free enzyme. Dinnellaetal[22] investigated the K_m and V_m which indicated K_m did not change and V_m decreased.

TABLE-1
KINETIC PARAMETERS OF FREE AND IMMOBILIZED CELLULASES

	V_m (μmol/mL min)	K_m (g/L)
Free cellulase	0.3801	11.577
Immobilized cellulase	0.2524	26.853

Note: pH = 4. 6, 0.2 mol/L acetate buffer, temperature = 40 ºC, time = 0.5 h, concentration of free cellulase 1 mg/mL.

Conclusion

An immobilized enzyme membrane with high performance was prepared by filtrating cellulase which was pre cross-linking with the glutaraldehyde on the dense surface of polysulfone membrane. The experimental results showed that ultrafiltration of enzyme solution improved. The enzyme loading density on membrane surface and cross-linking enhanced the enzyme stability. When the enzyme loading density was 0.2205 mg/cm^2, the maximum reaction rate per unit of membrane area (1.159 μmol Glucose/min cm^2) was achieved. Moreover, the performanceof enzymes immobilized has better pH and temperature stability In short, the cellulase immobilization onto the microporous polysulfone membrane surface by ultrafiltration and cross-linking with glutaraldehyde provide large loading density, good enzyme stability of biocatalytic membrane

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