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ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 54 (2008) 103–108
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Application of bacterial cellulose pellets in enzyme immobilization

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Received 20 September 2007; received in revised form 25 December 2007; accepted 27 December 2007
Available online 4 January 2008

Abstract

Over recent years, there has been a growing interest in the use of cellulose materials in bioprocessing technologies. Bacterial cellulose which is the pure cellulose has unique physical properties which differ from those of plant cellulose and has therefore attracted attention as a new functional material. The applications of bacterial cellulose rarely use the pellet type but it has potential in enzyme immobilization since pellet form is usually used in this field. In this research, Glucoamylase which is widely used in the food industry was immobilized on bacterial cellulose beads after testing using various activation procedures. The results showed that the epoxy method with glutaraldehyde coupling was the best method. After comparison of the different types of bacterial cellulose beads for glucoamylase immobilization, the wet bacterial cellulose beads of the smallest size (0.5–1.5 mm) were the best support. The immobilization of enzyme enhances its stability against changes in the pH value and temperature especially in the lower temperature region. The relative activity of the immobilized glucoamylase was still above 77% at pH 2.0 and it was the highest value in the literature. The relative activities were more than 68% in the lower temperature region even at 20 °C. Thus, bacterial cellulose beads are a practical potential support for the preparation of immobilized enzymes in industrial applications.

Keywords: Glucoamylase; Immobilization; Bacterial cellulose beads; Activation; Stability

1. Introduction

Starch is the most abundant form of storage polysaccharide in plants. Hence, its application is very important in the food industries such as in the production of oligo by starch hydrolysis. Acid splitting is the traditional method for the production of glucose syrup. However, the procedures are not suitable for industrial mass production since the products are rather complicated and require high purification costs. And since the cost of using starch-hydrolyzed enzymes is lower and its procedures are much simpler, it has become the main method in starch hydrolysis [1,2]. A number of enzymes are used in starch hydrolysis for the production of glucose, fructose or maltose. Glucoamylase is one of the key enzymes used for starch processing which has extensive uses in the manufacture of crystalline glucose or glucose syrup, either as soluble or immobilized enzymes. The enzyme hydrolyzes α-1,4- and -1,6-glycosidic linkages of starch to produce glucose [3]. In conventional enzymatic reactions, the soluble enzyme reacts with the substrate in the solution. After the completion of each batch of reactions, the enzymes are deactivated. Hence, the process would be more economical by using the immobilized systems since they allow the reuse of the enzymes. Immobilization of enzymes is generally carried out by adsorption or covalent coupling to solid matrices, as well as by entrapment or encapsulation in polymeric substances. Immobilization often results also in the improvement of enzyme stability under specific process conditions.

The application of the cellulose as a precursor for chemical modifications was exploited extensively even before its polymeric nature was determined and well understood [4]. In the present work, glucoamylase is immobilized on bacterial cellulose which is an indigenous food of South-East Asia. Bacterial cellulose has unique physical properties which differ from those of plant cellulose and has therefore attracted attention as a new functional material. Therefore, it has been receiving constant attention during the past decade for the production of bacterial cellulose with its unique structure and set of properties such as excellent mechanical strength, ultra-fine fiber, biodegradability, and high crystallinity [5–8]. Its widespread field of application includes foods, acoustic diaphragms, production of unusually strong paper, and medical applications such as wound dressings and artificial skins [6]. Acetobacter was used in the present work

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doi:10.1016/j.molcatb.2007.12.021

since it can produce ultrafine cellulose fibrils (50–80 nm in width and 3–8 nm in thickness). Cellulose fibrils are three dimensional network structures with a micrometer- to nanometer-scale [9].

Traditional production of bacterial cellulose was in stationary culture conditions and a thick, gelatinous membrane is accumulated on the surface of a culture medium, whereas under agitated culture conditions, produced cellulose is almost in the form of a fibrous suspension. The bacterial cellulose produced by the airlift reactor formed unique pellets, which were also much larger and different from the fibrous bacterial cellulose form produced in the stirred-tank reactor [10]. The applications of bacterial cellulose rarely use the pellet type but it has potential in enzyme immobilization since pellet form is usually used in this field. Therefore, the pellet type of bacterial cellulose was produced and tested for enzyme immobilization in the present work. The large-scale production of the pellet form of bacterial cellulose in the fermentor was established recently using the conventional airlift reactor in 50 L [11] and modified airlift reactor in 20 L [12]. Thus, the application of bacterial cellulose pellet is expected to expand even more.

The immobilization of glucoamylase on different types of bacterial cellulose beads was investigated in the present work. Bacterial cellulose beads were produced by the strain of *Acetobacter xylinum* in a shaking flask with baffle. A range of immobilization chemistries were also examined for active bacterial cellulose with the aim of maximizing both the amount of immobilized biocatalyst and the retention of enzymatic activity. Thermal, pH, and storage stability of immobilized enzymes were also studied and compared with free enzymes. The results are expected to have practical importance for further applications.

2. Materials and methods

2.1. Materials

Glucoamylase (amyloglucosidase, exo-1,4-α-glucosidase, EC 3.2.1.3, from *Aspergillus niger*), soluble starch (from potato, as substrate in determining enzyme activity according to Zulkowsky) and 3,5-dinitro salicylic acid were purchased from Sigma Chemical Co. Glucose and peptone were obtained from Merck Co. Ltd. Germany while yeast extract was obtained from Sigma Chemical Co. For the activated reagents, 1,4-butanediol diglycidyl ether and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; $C_8H_{17}N_3{\cdot}HCl$) were obtained from Sigma Chemical Co., glutaraldehyde was purchased from Fluka. The other chemicals were of analytical grade.

2.2. Microorganism and cultivation conditions

The microorganism, *A. xylinum* subsp. *Sucrofermentans* BPR2001, used in this study was purchased from Japan Collection of Microorganisms (RIKEN, Saitama, Japan). The culture medium for its maintenance contained (%, w/v): mannitol, 2.5; yeast extract, 0.5; peptone, 0.3; agar, 1.5 at pH 5.5. The culture medium used for the production of bacterial cellulose beads was the Hestrin & Schramn (HS) medium consisting of 2.0% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.27% (w/v) $Na_2HPO_4{\cdot}12H_2O$ and 0.115% (w/v) citric acid monohydrate [12]. Prior to sterilization at 121 °C, the pH value of the medium was adjusted to 5.0. The organisms were grown in a 500 mL flask containing 300 mL of the HS medium and then cultivated at 30 °C and 120 rpm. The cultivation time was at least 48 h and based on the size of bacterial cellulose beads for the desired experiments.

2.3. Preparation of bacterial cellulose beads

To obtain the bacterial cellulose, samples from the culture broth were centrifuged at 9100 rpm for 30 min. The cellulose beads were obtained by dissolving the cells with 0.1 N NaOH at 90 °C for 30 min and then washed twice with deionized water.

2.4. Activation

2.4.1. Epoxy method (M1) [13]

The bacterial cellulose beads (1 g) were suspended in 1.5 mL of 1 M NaOH and then 0.2 mL of 1,4-butanediol diglycidyl ether was added. The mixture was stirred at 60 °C for 2 h and the final product was washed successively with distilled water.

2.4.2. Epoxy with EDC coupling method (M2)

The bacterial cellulose beads (1 g) produced by the epoxy method using 1,4-butanediol diglycidyl ether were mixed with 2.5 mL of distilled water and 1 g glycine at 60 °C and pH 12 for 2 h. After that, the beads were activated with 3 mL of 0.1% EDC at 25 °C for 1 h. Finally, the activated bacterial cellulose beads were washed successively with distilled water.

2.4.3. Epoxy with glutaraldehyde coupling method (M3)

The bacterial cellulose beads (1 g) produced by the epoxy method using 1,4-butanediol diglycidyl ether were mixed with 0.5 mL of distilled water and 0.5 mL of 30% (w/v) ammonia. The mixture was stirred at 60 °C for 2 h. After washing with distilled water, the beads were mixed with 1 mL of 25% (w/v) aqueous glutaraldehyde for 2 h. The glutaraldehyde-activated bacterial cellulose beads were washed successively with distilled water.

2.4.4. Epoxy with glutaraldehyde and EDC coupling method (M4)

The bacterial cellulose beads (1 g) produced by the glutaraldehyde method was mixed with 3 mL of 0.1% EDC and stirred for 1 h at room temperature. The product was washed successively with distilled water.

2.5. Immobilization of glucoamylase

Glucoamylase immobilization was carried out by adding the bacterial cellulose beads (1 g) to a suspension of 3 mL glucoamylase (0.1%) in 5 mM sodium phosphate at pH 4.5 and 25 °C for 60 min. After immobilization, the derivatives were washed with distilled water.

2.6. Estimation of enzyme activities

The activity of the free and the immobilized glucoamylase were determined by estimating the amount of glucose produced by enzyme. The hydrolysis reactions were carried out in the phosphate buffer with 1% of the substrate. In present study, starch was chosen as the substrate. After adjusting the pH to 4.5, the mixture was incubated at 60 °C for 3 min. The liberated glucose was measured using DNS (3,5-dinitrosalicylic acid) method. One unit of enzyme is defined as the amount of enzyme which releases reducing carbohydrates equivalent to 1 μmol glucose from soluble starch per min.

The kinetic parameters (Michaelis constant K_m and maximum rate V_{max}) were calculated by measuring the rates of reaction at various substrate concentrations. The values were substituted into the Hanes–Woolf equation to obtain K_m and V_{max}.

2.7. pH and thermal stability of immobilized glucoamylase

The pH stability of the immobilized glucoamylase was studied by incubating the immobilized enzyme at 25 °C in buffers of varying pH (3.5–6.5) for 1 h and then determining the hydrolytic activity at the optimum pH and temperature. Relative activities were calculated as the ratio of the activity of immobilized enzyme after incubation to the activity at the optimum reaction pH. The thermal stability of glucoamylase was tested by incubating the immobilized enzyme at varying temperatures (20–90 °C) and determining the activity at its optimum reaction temperature. Relative activities were calculated as mentioned above and plotted against temperature.

3. Results and discussions

3.1. Comparison of different activate methods

Four activate methods were selected from literature to investigate whether the enzymes could be most effectively immobilized on solid phases for practical applications or not. Among the methods, the glutaraldehyde and EDC coupling methods were derived from the epoxy method and the results are shown in Table 1. The epoxy method with glutaraldehyde coupling was the best method and its relative activity was at least 15% more than the others. Therefore, M3 procedure was chosen in the following experiments since its highest relative activity after immobilization.

Table 1
Relative activities of immobilized enzymes for the different activated methods

Method	Relative activity (%)
M1 (Epoxy method)	85.3
M2 (Epoxy with EDC coupling method)	68.9
M3 (Epoxy with Glutaraldehyde coupling method)	100
M4 (Epoxy with Glutaraldehyde and EDC coupling method)	82.6

Table 2
Effects of particle size on the relative activity of immobilized glucoamylase

Particle size (mm)	Relative activity (%)	Activity (U/g-carrier)
4–5	32	15.2
2–3	42	20.0
0.5–1.5	100	47.6

3.2. Effects of drying condition and particle size

The cultivation of *A. xylinum* produced different sizes of bacterial cellulose beads based on the cultivation time. Hence, the preparation of bacterial cellulose beads was discussed first. After a suitable cultivation time, particle size with different ranges 0.5–1.5, 2–3 and 4–5 mm were selected and used to test the immobilization of the enzyme. The results are shown in Table 2. The relative activity was significantly higher when the smaller beads were used. The results were logical since the smaller beads provided a greater surface area and larger functional groups to connect with the enzymes.

The bacterial cellulose beads with different water contents were also investigated. The three kinds of bacterial cellulose beads, namely, wet, simply dried at room temperature for 24 h, and oven dried at 50 °C for 24 h, were tested for their activities after immobilization of the enzyme. From the results shown in Table 3, the wet particles showed a significantly higher relative activity than the others. The immobilized efficiency calculated as the ratio of bound protein (measured activity) and amount of protein (activity) used for immobilization is about 38.3% which is common value in chemical adsorption.

Thus, the wet bacterial cellulose beads with the smallest size were used in the following experiments to test the characteristics of the immobilized enzyme.

3.3. The time of immobilization

Experiments were performed using different immobilization times from 15 min to 120 min with intervals of 15 min and the results are shown in Fig. 1. The maximum relative activity was

Table 3
Effects of drying condition on the relative activity of immobilized glucoamylase

Drying condition	Dry weight (g)	Syneresis (%)	Relative activity (%)	Activity (U/g-carrier)
Wet	1	100	100	47.6
Drying at room temperature for 24 h	0.0135	98.7	52	24.8
Drying at 50 °C for 24 h	0.0103	99.0	46	21.9

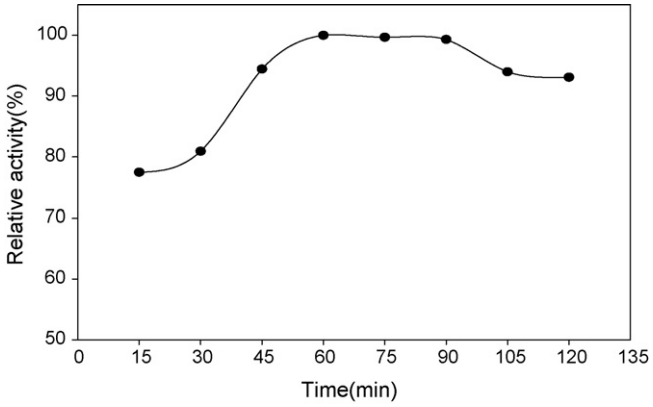

Fig. 1. Effect of the immobilization time on the relative activity of immobilized glucoamylase.

achieved in 60 min of immobilization time. Above that time, the relative activity would slightly decrease. Hence, 1 h was selected as the best length of time for enzyme immobilization.

3.4. Effect of pH on enzyme activity

The effect of pH on the relative activity of glucoamylase immobilized on the bacterial cellulose beads was studied by varying the pH of the reaction medium from 2 to 6.5 at an interval of 0.5 and the pH profile is shown in Fig. 2. After the immobilization, the optimum pH was slightly shifted from 5 to 4.5 when compared to the free enzyme. The similar tendency for pH shift to acid was found in other research [14,15]. The better operating range, i.e. when the relative activities of the enzyme were above 90%, was also slightly increased from 3.5–5.5 to 3.0–5.5. In other words, the immobilized enzyme activity was better in acid when compared to the free enzyme. Moreover, the relative activities of the immobilized glucoamylase were still above 77% at pH 2.0. When compared with the highest value 83% at pH 2.5 in the recent research by Chang and Juang [15], it is also the same value, i.e. 83%. Therefore, the immobilization enzymes in bacterial cellulose have a broader pH range of high activity than free enzymes.

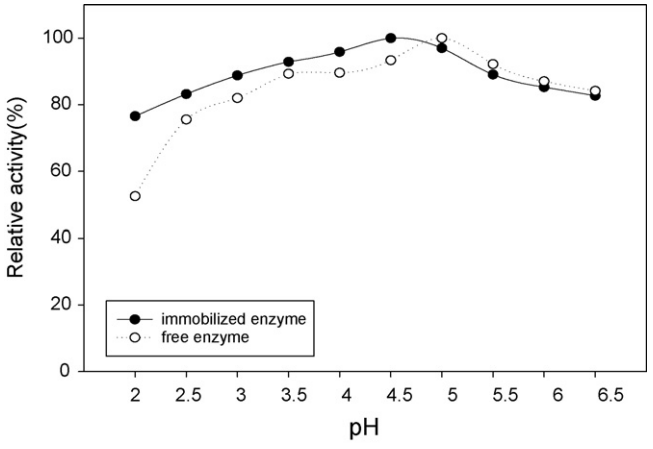

Fig. 2. Effect of substrate pH on the relative activity of free and immobilized glucoamylase.

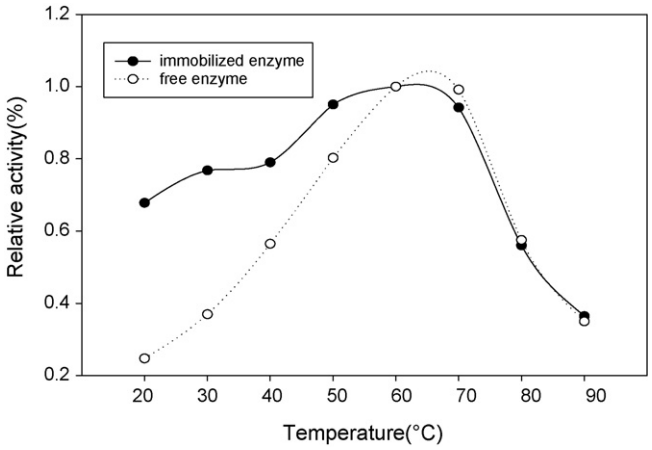

Fig. 3. Effect of reaction temperature on the relative activity of free and immobilized glucoamylase.

3.5. Effect of temperature on enzyme activity

The temperature dependence of the hydrolytic activity of free and immobilized glucoamylase is shown in Fig. 3. The optimum reaction temperature of the glucoamylase was at 60 °C for both cases. The better operating range, i.e. when the relative activities of the enzyme were above 90%, was at 50–70 °C by immobilization which was wider than that of the free enzyme (60–70 °C). The 10 °C decrease in the optimum activity temperature combined with thermal stability exhibited by the bacterial cellulose beads was an interesting finding of the present work. A similar behavior was also obtained recently [15–17]. The industrial applications of glucoamylases no longer required temperatures around 60–70 °C to reach the optimum catalytic activity temperature. Moreover, the relative activities of the immobilized glucoamylase were still above 68% at 20 °C in the present work. That means that the relative activities were more than 68% in the temperature range of 20–70 °C. This result is closed to the highest value 74% in the recent research by Chang and Juang [15]. This ability takes the advantage of the energy consumption in industry process since the substrate will not be needed to preheat.

The fast decrease in the relative activities at the high temperature range for both cases was due to the thermal denaturation. Thus, the immobilized glucoamylase on the bacterial cellulose beads could increase the enzyme activation but could not protect the enzyme for the thermal denature effect.

3.6. Stability of the immobilized enzyme

The stability of the relative activity of the free and the immobilized glucoamylase with pH is shown in Fig. 4. Free enzyme was stable at pH 4 and the relative activities were maintained at 90% in the pH range of 3–6. The immobilized glucoamylase was stable at pH 7 and the relative activity was maintained above 80% in the pH range of 5–7. This result indicated that the enzyme activity had more influence on the pH after immobilization.

The thermal stabilities of the free and the immobilized glucoamylase in terms of the relative activities are compared in

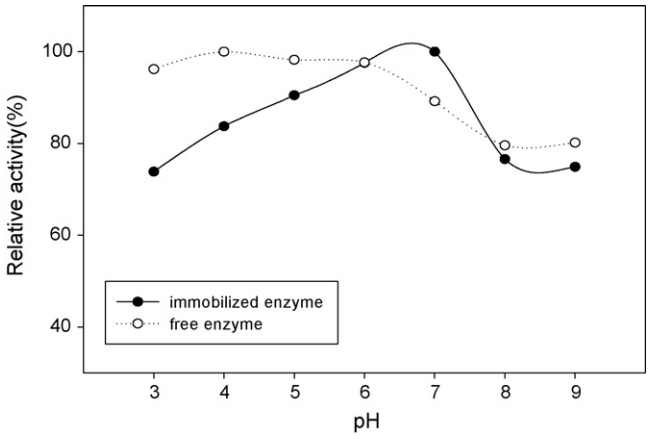

Fig. 4. pH stability of free and immobilized glucoamylase.

Fig. 5. Thermal stability of free and immobilized glucoamylase.

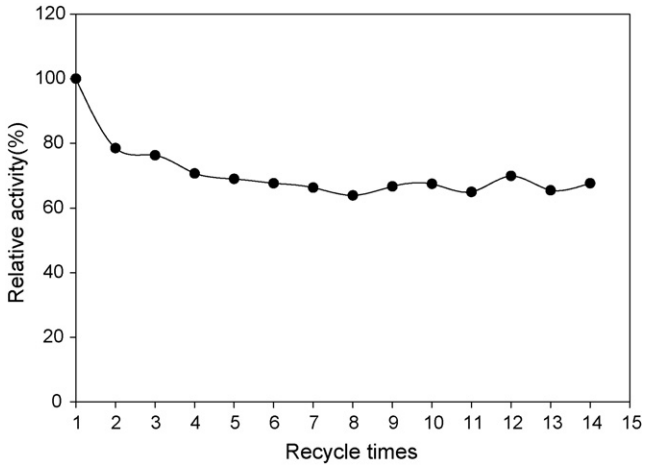

Fig. 6. Effect of repeated use on the relative activity of immobilized glucoamylase.

Fig. 5. The enzyme was found to be stable up to a temperature of 50 °C for the free enzyme, and up to 60 °C for the immobilized enzyme which was slightly better than the free enzyme. Hence, the effect of thermal deactivation for the immobilized enzyme was not significant as compared to the free enzyme state.

3.7. Reuse stability of immobilized enzymes

Glucoamylase immobilized in the bacterial cellulose beads was used repeatedly to hydrolyze starch since reusability was important for repeated applications in a batch or a continuous reactor. The relative activity of the immobilized glucoamylase when repeatedly used is shown in Fig. 6. After 14 repeated usages, the immobilized glucoamylase retained 60% of the residual activity. The relative activity that significantly decreased by at least 20% after the first reaction could be due to the imperfect washing step. The activity of the repeated usage of immobilized glucoamylase was almost constant from cycle 4 to 14. Thus, the immobilized enzyme has a potential for industrial applications.

3.8. Kinetics of hydrolysis

The Michaelis–Menten kinetics of the hydrolytic activity of the free and the immobilized glucoamylase was investigated by initial rate method. After measuring the changes of product concentrations against time at different initial substrate (soluble starch) concentrations, the initial production rate can be calculated. The Michaelis constant, K_m, and the maximum reaction velocity, V_{max}, were evaluated from the double reciprocal plot of initial production rate and substrate (Lineweaver–Burk plots).

The V_{max} value of 1.75 g/L/min exhibited by the immobilized glucoamylase to the bacterial cellulose beads was lower than that of the free enzyme (2.62 mg/mL/min). The K_m value determined for the immobilized glucoamylase (3.27 mg/mL) was slightly increased than that of the free glucoamylase (3.03 mg/mL), and indicated a similar affinity behavior toward the substrate. The phenomena in the K_m value shows that the structure of enzyme does not change in this immobilized support. This result was similar as the immobilization of glucoamylase in the other natural polymers such as chitosan-clay composite [15], activated charcoal [17] and montmorillonite [18].

4. Conclusions

Glucoamylase was immobilized to bacterial cellulose beads with the use of some activated methods and the epoxy method with glutaraldehyde coupling was the best one. The wet bacterial cellulose beads with the smallest size were the best choice for the different drying conditions and particle sizes. The immobilized glucoamylase enhanced the enzyme abilities against changes in the pH value and temperature especially in the lower temperature region. The relative activity of the immobilized glucoamylase was still above 77% at pH 2.0 and it was the highest value in the literature. The relative activities were more than 68% in the lower temperature region even at 20 °C. Therefore, the bacterial cellulose beads are a promising support for the preparation of immobilized glucoamylase for industrial applications.

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