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Immobilization of Fungal β-Glucosidase on Silica Gel and Kaolin Carriers

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Abstract β-Glucosidase is a key enzyme in the hydrolysis of cellulose for producing feedstock glucose for various industrial processes. Reuse of enzyme through immobilization can significantly improve the economic characteristics of the process. Immobilization of the fungal β-glucosidase by covalent binding and physical adsorption on silica gel and kaolin was conducted for consequent application of these procedures in large-scale industrial processes. Different immobilization parameters (incubation time, ionic strength, pH, enzyme/support ratio, glutaric aldehyde concentration, etc.) were evaluated for their effect on the thermal stability of the immobilized enzyme. It was shown that the immobilized enzyme activity is stable at 50 °C over 8 days. It has also been shown that in the case of immobilization on kaolin, approximately 95% of the initial enzyme was immobilized onto support, and loss of activity was not observed. However, covalent binding of the enzyme to silica gel brings significant loss of enzyme activity, and only 35% of activity was preserved. In the case of physical adsorption on kaolin, gradual desorption of enzyme takes place. To prevent this process, we have carried out chemical modification of the protein. As a result, after repeated washings, enzyme desorption from kaolin has been reduced from 75 to 20–25% loss.

Keywords β-glucosidase · Immobilization · Silica gel · Kaolin · Thermostability · Corn stover · Enzymatic hydrolysis

Introduction

β-Glucosidase is a key enzyme in the process of enzymatic hydrolysis of cellulose to glucose, functioning primarily to hydrolyze cellobiose to two glucoses [1]. β-Glucosidase

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can also demonstrate activity on cello-oligomers, and although the activity varies, it generally decreases with increasing chain length. The cellulase complex from the main fungal producers normally contains small amounts of β-glucosidase, which may limit the overall cellulose hydrolysis rate, as cellobiose inhibits activity of other enzymes of cellulose complex [2]. β-Glucosidase is also subject to end-product inhibition, with removal of glucose significantly enhancing the enzyme activity rate.

The cost of enzyme preparations has been decreasing in recent years; however, it continues to affect considerably the price of ethanol obtained from cellulosic raw materials. Increased enzymatic hydrolysis efficiency is one way to reduce the enzyme cost in bioethanol production. Another method is enzyme recycle and reuse. Immobilization of biocatalysts allows for their economic reuse and development of continuous bioprocess. Although immobilization poses problems of substrate accessibility and binding for most endo- and exocellulases, β-glucosidase exhibits characteristics amenable to immobilization, such as activity on soluble substrates and the lack of a carbohydrate-binding module. Among the possible approaches, immobilization of β-glucosidase is one prospective solution to the problem.

The variety of protein immobilization methods can be reduced to two main approaches: physical adsorption and covalent binding with the carrier. Both approaches have their advantages and shortcomings [3–6]. The advantage of adsorption is simplicity, as usually no cross-linking reagents or activation steps are required. As a result, adsorption is cheap, easily carried out, and tends to be less destructive to the enzyme than chemical means of the attachments. The shortcoming of physical binding is non-stability of the bonds between enzyme and carrier. In covalent binding, the bonds between carrier and protein are very firm, resulting in a highly stable conjugate, but very often the enzyme activity drops significantly.

We have examined several carrier matrices for their ability to immobilize β-glucosidase, including activated charcoal, nylon, chitosan, bentonite, kaolin, silica gel, and titanium dioxide. The results indicated that immobilization by covalent binding on silica gel and by physical adsorption on kaolin were the most prospective methods for improvement of the economic parameters of enzymatic hydrolysis of cellulose. Here, we report on these two supports.

Materials and Methods

Enzyme Assays and Stability Determination

Determination of glucose was carried out by glucose oxidase/peroxidase method [7]. The activity of β-glucosidase was determined as follows: the enzyme preparation was diluted 10- to 50-fold depending on the initial activity. Substrate used was a 1% (*v*/*w*) solution of cellobiose (Sigma) in 0.05 M sodium acetate buffer pH 5.0. Fifty microliters of enzyme solution were added to 5 ml of substrate incubated at 45 °C. After 30 min, test tubes were placed into a boiling water bath for 10 min. Concentration of glucose formed in the process of reaction was determined by the method mentioned above. Activity units of β-glucosidase were expressed as μMol of glucose/min ml^{-1} of enzyme preparation.

The thermal stability of free and immobilized enzyme preparations was determined by incubation at 70 °C, pH 5.0 (0.05 M sodium acetate buffer). Every 10 min, aliquots were taken, and β-glucosidase activity was determined.

Enzyme Immobilization

To remove low molecular weight compounds that interfere with enzyme activity, 20 ml of Novozym 188 was dialyzed against 1.5 l of 0.15 M solution of sodium acetate buffer (pH 5.5) for 2 days. After 24 h, the solution was replaced by fresh buffer. To examine the effect of hydrophobicity of the β-glucosidase on non-covalent immobilization, a subset of β-glucosidase was acetylated. Protein acetylation was carried out by acetic anhydride by a modified method according to Ansari [8]. Four milliliters of acetic anhydride was added to 65 ml of the final solution of the enzyme for acetylation of free amino groups of lysine and arginine. The mixture was incubated at 25 °C for 2 h. After acetylation, the obtained preparation was dialyzed against 1 l of 3 M solution of sodium acetate for deacetylation of other amino acids. Dialysis medium was replaced twice during 2 days.

Silanization of silica gel was carried out according to Walters [9]. Silica gel 40/100 was silanized by (γ-aminopropiltriethoxysilane). Procedure involved 5 h boiling of 10% silica gel suspension with 10% silane solution. The silica gel was then washed exhaustively by decantation to remove excess of silane. The silanized silica gel was activated by glutaric aldehyde at 37 °C for 24 h with periodic mixing. As increased concentrations of glutaric aldehyde bring to decreased activity of immobilized enzyme, we have used glutaric aldehyde in final concentration 1.5%. Exhaustive decantation of supernatant was conducted to remove excess of glutaric aldehyde. Finally, silica gel was suspended in 0.01 M phosphate buffer pH 6.8, containing 0.05 M NaCl. Conjugation of the β-glucosidase to activated silica gel was conducted. Conjugation was conducted at 37 °C during 24 h with periodic mixing in 0.01 M phosphate buffer pH 6.8 NaCl 0.05 M. The amount of enzyme was varied from 3.46 to 17.3 mg/ml of silica gel suspension.

Kaolin granules were prepared from dry powdered kaolin after hydration to 45–48% moisture. The obtained paste was rubbed through a metal sieve with a mesh diameter 0.6 mm to make granules with the maximal length of 1 mm and baked at 550 °C for 2 h. The kaolin granules were washed in aliquots of 1 g in 50 ml distilled H_2O by centrifugation. After washing several times, dialyzed Novozym 188 was added to 1 ml of the kaolin suspension in a final buffer concentration of 50 mM sodium acetate. The amount of enzyme was varied from 3.46 to 17.3 mg/ml of kaolin suspension. After incubation during 2 h at 40 °C with mixing, the suspension was centrifuged for removal of non-bound protein,and activity of the absorbed protein was determined by standard glucose oxidase/peroxidase method.

Determination of Immobilization Parameters

Retained Enzyme After binding of enzyme to carrier, the mix was washed by centrifugation to remove unbound protein. The amount of enzyme bound to each carrier was determined by difference after determination of residual protein content from the supernatants after washing the carrier/enzyme conjugate.

K_m Measurement Approximate K_m values were determined using a range of cellobiose concentrations in 50 mM acetate buffer, pH 5.0. Enzyme was added to the pre-equilibrated substrate, and buffer and glucose were determined after reaction by the method above. As reproducible aliquots were difficult to obtain for immobilized enzyme samples, the samples were dried to determine dry weight, and the amount of enzyme present in the reaction was calculated based on the retained enzyme value for each carrier and normalized to 10 mg of enzyme.

Thermal Stability The thermal stability of the β-glucosidase was determined by measuring activity over time after incubation at 70 °C. Samples were taken at 10 min intervals, cooled on ice, and activity determined on cellobiose as above. The thermal stability of β-glucosidase from Novozym188 was determined for free native enzyme, bound native enzyme, free acetylated enzyme, and bound acetylated enzyme.

Desorption The procedure of evaluation of enzyme desorption from the carrier was as follows: after incubating the enzyme with the carrier in corresponding conditions, the suspension was centrifuged. The precipitate was then washed by buffer solution and centrifuged once more. This procedure was repeated three times by tenfold quantity of the buffer. Then, we joined all the portions of supernatants; after which, we measured the activity of the immobilized enzyme and the activity of the supernatant. We have also measured preliminarily the activity of the enzyme solution that was used for immobilization. Based on the correlation between activities, we calculated the level of desorption of the enzyme from the carrier.

Fluidized Bed Reactor To determine the amenability of immobilized enzyme to process conditions, a fluidized bed reactor was used to measure continuous glucose production from cellobiose. We used granulated kaolin with immobilized acetylated β-glucosidase in the fluidized bed reactor. A thermostatically controlled column with a diameter of 1.6 cm and a height of 18 cm was used as the reactor. After filling, the part of the column filled with kaolin granules made 30 ml. The column was preliminary washed by the buffer during 24 h to wash out the non-absorbed enzyme and fine part of the carrier, after which, we measured the activity of β-glucosidase in the effluent solution. We assessed the level of conversion of the substrate into glucose by running 100 g/l cellobiose solution through the column in 50 mM sodium acetate buffer solution. The correlation between the level of conversion of the substrate and the velocity of running of the solution through the column was defined by varying the flow rate through the column.

Digestion of Pretreated Corn Stover

Pretreated corn stover was graciously provided by the National Renewable Energy Laboratory (Golden, Colorado). The stover was pretreated at 190 °C at 30% solids with 0.048 g H_2SO_4/g dry biomass. The reactor residence time was approximately 1 min. After pretreatment, the composition of the corn stover was 59.9% glucan, 4.74% xylan, 0.69% arabinan, 0.2% mannan, 0.47% galactan, 25.53% lignin, 2.44% protein, and 3.4% ash. Digestions were carried out using a commercial cellulase (Spezyme CP, Genencor, Intl.) augmented with β-glucosidase (Novozym 188, Novozymes Inc., Davis, CA., 250 CBU/g) immobilized on kaolin as above. Protein was determined by Biuret method according to Gornall et al. [10] or dye binding method according to Bradford [11].

Enzymatic hydrolysis of pretreated corn stover was conducted at 10% solid concentration in 50 mM sodium acetate buffer (pH 5.0) in Erlenmeyer flasks. Into each flask was added 50 ml of 10% PCS suspension, and 0.5 ml of the enzyme solution was diluted to the level that provides necessary enzyme activity.

Spezyme CP was loaded at 5 FPU/g cellulose for cellulase-only digestions and 2.5 FPU/g cellulose when assayed with β-glucosidase present. β-glucosidase was added at 15 CBU/g cellulose. Flasks were preincubated at 45 °C on the rotary shaker at 150 rpm for 10 min, and

the enzymes were added to start the hydrolysis. Aliquots of 2 ml were taken at different time points, immediately chilled on ice, and centrifuged at 5,000×*g* for 10 min. Glucose analyses were performed on the resultant supernatants.

To obtain statistically valid data, all the experiments have been carried out four times for each series, and within each series, the experiments were replicated five times for each investigated parameter. The results of replications were averaged. The deviations between the results of replications did not generally exceed 6%.

Results and Discussion

Choice of Immobilization Method

We have studied various carriers that are applicable for immobilization of β-glucosidase (activated charcoal, chitosan, nylon, bentonite, kaolin, silica gel, etc.) and determined several parameters essential for practical application of the immobilized enzyme. Initial work using almond β-glucosidase evaluated the binding parameters for the various carriers tested. Taking into account the main parameters, we have found that covalent binding with silica gel and physical adsorption on kaolin granules are the most efficient methods of immobilization. Table 1 presents the data of immobilization of β-glucosidase on the indicated carriers.

As Table 1 demonstrates, silica gel is superior to kaolin in some parameters (K_m, thermostability, desorption); however, the relative costs of the two carriers and superiority of kaolin in enzyme binding capacity and retention suggest that kaolin is much more economically favored for large-scale processes compared to silica gel. The primary drawback to kaolin is its propensity to lose enzyme by desorption over long exposure times. Taking into account the fact that desorption of the enzyme from the carrier in the process of fermentation may become a determinative factor, we made an attempt to improve this parameter by changing the hydrophobicity of the enzyme preparation. We carried out acetylation of the enzyme preparation and compared the native and acetylated preparations with respect to several parameters. Table 2 demonstrates data on the level of binding of the enzyme with the carrier. Acetylation of β-glucosidase, which is aimed at increasing the hydrophobicity of protein molecule surface, increases the level of binding of the enzyme with the carrier.

The results indicate that acetylation of the enzyme considerably improves immobilization stability of the process. Acetylation of the enzyme reduces enzyme desorption resulting

Table 1 Comparison of immobilization methods.

Enzyme immobilization parameter	Kaolin	Silica gel
K_m, (mM cellobiose)	1.8	10
Enzyme/support ratio, mg/g	140	55
Retained, %	95	35
Thermostability, min[a]	11	21
Desorption, %[b]	22	0

[a] Enzyme half-life at t=70 °C

[b] Desorbed activity was determined after washing of column by 200× volume of buffer solution.

Table 2 Adsorption of native and acetylated preparation of β-glucosidase on kaolin.

Conjugates	Activity (initial)	Activity (100× wash)	Activity (300× wash)	Activity (500× wash)
Kaolin/native enzyme	0.63	0.56	0.46	0.44
Kaolin/acetylated enzyme	0.69	0.64	0.62	0.61

from washing by greater than fourfold compared to native protein. As the activity of the acetylated enzyme is equivalent to native (or slightly better), this represents an important step in enabling the reuse and recycle of β-glucosidase in biomass processing. The kaolin immobilized acetylated β-glucosidase was also evaluated in a fluidized bed reactor for continuous production of glucose from cellobiose. The powdered kaolin was unusable for this purpose, as it either diluted out of the column (upflow) or packed tightly to very high back pressure (downflow); the granulated kaolin was used. The two kaolin forms showed equivalent capacities and activities of bound enzyme, although the granulated form exhibited a slightly higher binding capacity. After washing the column for 24 h, β-glucosidase activity was determined for the effluent. We did not observe any appreciable enzyme desorption from the column. During velocity experiments with cellobiose solutions, the column performed at a stable rate until the velocity 40 ml/h, at which point, the residence time of the cellobiose apparently exceeded the reaction rate and glucose production decreased (Fig. 1).

Comparative Analysis of Thermostability of Native and Acetylated Enzyme

Another essential factor is the change of thermostability of the enzyme as a consequence of chemical modification and immobilization. We have determined the thermostability of free and immobilized forms of native and acetylated β-glucosidase. The results of these measurements are presented in Figs. 2 and 3.

As the results presented in Figs. 2 and 3 indicate, thermostability of free, native β-glucosidase is quite high, and after the immobilization on kaolin, it decreases slightly. Acetylation of the enzyme causes substantial decrease of thermostability. Thus, if the native enzyme preserves approximately 45% of its initial activity at 70 °C during 10 min, the acetylated enzyme at the same conditions preserves only 18% of its initial activity. Although acetylation of β-glucosidase decreased the thermostability of the enzyme

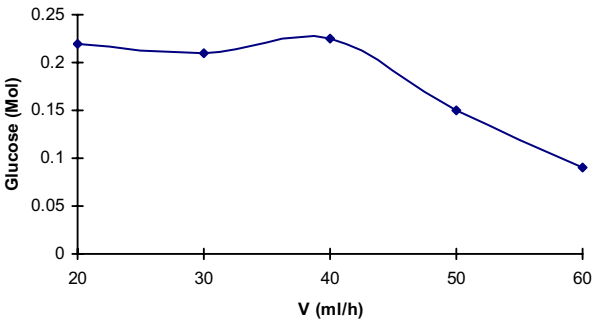

Fig. 1 Glucose production in a fluidized bed reactor by immobilized β-glucosidase

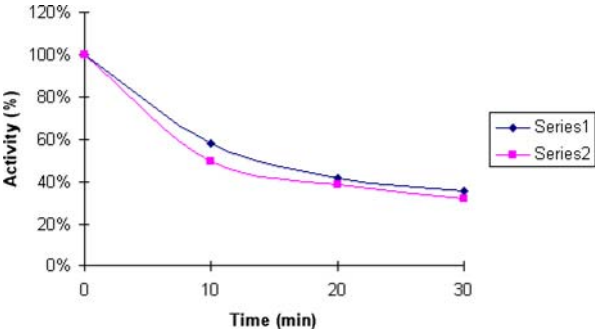

Fig. 2 Thermostability of β-glucosidase from Novozym 188 preparation in free and immobilized forms. *1* Free enzyme, *2* immobilized enzyme, *t* 70 °C, pH 5.0

compared to the native enzyme, immobilization restored much of the thermostability to near-native levels.

Chemical modification of the enzyme and its immobilization may cause either increase or decrease of thermostability [12–14] depending on the enzyme, nature of the carrier, and the method of immobilization. As the data from Nagamoto et al. [15] indicates, thermostability of β-glucosidase immobilized on chitosan increases, and the enzyme works successfully at 70 °C during 12 h. Such considerable increase of thermostability that we observed is due to the covalent binding of the enzyme and the carrier.

Taking into account the fact that acetylated enzyme adsorbs on the carrier more firmly, acetylation can be regarded as a possible way to improve the immobilization characteristics of β-glucosidase.

Enzymatic Hydrolysis of Pretreated Corn Stover

To evaluate the practical application of immobilized β-glucosidase for hydrolysis of pretreated corn stover, we have carried out enzymatic hydrolysis in flasks with the use of cellulase and immobilized β-glucosidase. Taking into account the fact that Spezyme CP contains a certain quantity of β-glucosidase, we used reduced quantities of Spezyme CP in our experiments—5 U/g of glucan instead of normally used 15–40 U/1 g of glucan. This allowed us to better evaluate the effect of immobilized β-glucosidase in the process of hydrolysis of corn stover. The obtained results are presented in Fig. 4.

As Fig. 4 shows, the addition of immobilized β-glucosidase to the preparation Spezyme accelerates substantially the production of glucose and increases the level of hydrolysis. Taking into account the contents of glucan in PCS and basing on the obtained results, we

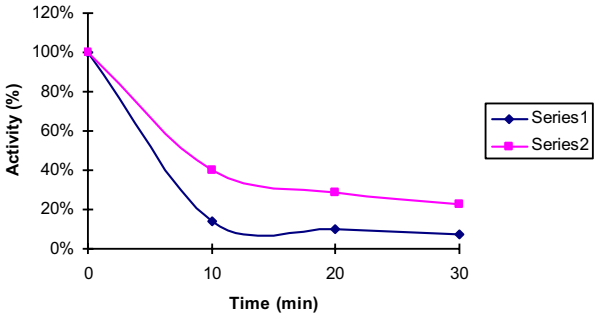

Fig. 3 Thermostability of acetylated β-glucosidase from Novozym 188 preparation in free and immobilized forms. *1* Free enzyme, 2 immobilized enzyme, *t* 70 °C, pH 5.0

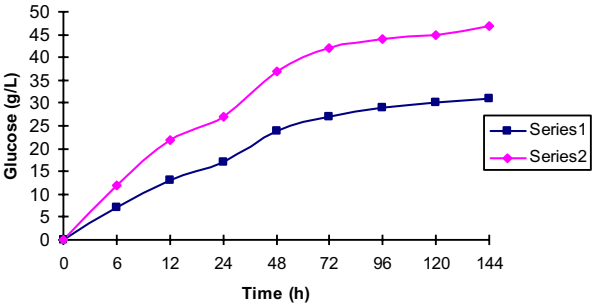

Fig. 4 Glucose formation from washed pretreated corn stover (PCS) by kaolin-immobilized beta-glucosidase (Novozym188) and cellulase. Pretreated corn stover 10% (*w*/*v*) solids, *t* 5 °C, pH 5.0. Series (1) 5 FPU cellulose/g glucan. Series (2) 5 FPU cellulase per g glucan+15 CBU β

can see that when using Spezyme, the level of conversion of glucan into glucose reaches about 50% after 144 h, while the addition of immobilized β-glucosidase enhances the conversion to 79%. For comparison, we can present the data [16, 17] where the authors studied possibility of application of immobilized β-glucosidase for the winemaking industry. The stability of β-glucosidase immobilized on chitosan pellets and Duolite A-568 resin was studied. In the case of chitosan, authors point out considerable stability of the enzyme (half-life 1.2 years). According to our data at 50°C, the activity of β-glucosidase immobilized on kaolin does not change notably for 300 h (observed time). Consequently, in the process of simultaneous saccharification and fermentation, i.e., at the temperature 35 °C, the immobilized enzyme is reusable at least five to six times during 1/5–2 months. Although our indices for time span of the enzyme usability are lower than those from the literature, it should be taken into notice that in the mentioned work, the immobilization carried out by covalent binding. If we take into account all the factors that determine the costs of enzymatic hydrolysis, we will see that our approach has certain advantages.

During enzyme hydrolysis of biomass, Spezyme CP supplemented with immobilized β-glucosidase gave satisfactory results of saccharification of pretreated corn stover, increasing the digestion rate and extent while enabling the reduction of cellulase loading. Thus, immobilization of β-glucosidase on kaolin can be regarded as a prospective method for multiple use of the enzyme at ethanol production from cellulose-containing raw materials.

Conclusion

Studies of immobilization on different carriers indicate that immobilization on silica gel or kaolin is the most prospective for application in the future, although the potential economic benefits appear much more favorable for kaolin. Comparison of immobilization characteristics of β-glucosidase immobilized on silica gel and on kaolin has shown that the kaolin method has more advantages, as it is considerably cheaper and more efficient. The feasibility of preliminary acetylation of the enzyme preparation requires further investigation. We have to take into consideration additional expenses necessary for acetylation and the issue of the decrease of the enzyme activity after acetylation.

These preliminary studies indicate a good potential for the use of immobilized β-glucosidase in the enzymatic conversion of biomass to sugars. Significant work, however, needs to be continued to evaluate efficacies under more process-relevant conditions.

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