Stabilization of the Cellulase Enzyme Complex as Enzyme Nanoparticle

Imre Hegedüs · Jenő Hancsók · Endre Nagy

Abstract The native Celluclast BG cellulase enzyme complex consists of different enzymes which can also degrade great substrate molecules as native celluloses. This enzyme complex has been covered by a very thin, a few nanometers thick, polymer layer, in order to improve its stability. It has been proved that the polymer layer around the enzyme molecules does not hinder the digestion as great substrates as crystalline cellulose polymer. The stability of the prepared enzyme nanoparticles (PE) could significantly be increased comparing to that of the native one what was proved by results of the total cellulose activity measured. The pretreated enzyme complex holds its activity often a few magnitudes of orders longer in time than that of the native enzyme complex (enzyme without pretreatment). It retains its activity at least ten times longer than that of the native one, at a temperature range between 20 and 37 °C. The pretreated enzyme complex can have about 50 % of its original activity during 12 h of incubation at even 80 °C, while the native cellulase one totally lost it during 6 h incubation time. The activity of PE has not been significantly reduced even at extreme pH values, namely in the pH range of 1.5 to 12.

Keywords Enzyme activity · Enzyme stabilization · Single enzyme nanoparticle · Enzyme complex · Cellulase

Abbreviations

AOT   Sodium bis(2-ethylhexyl) sulfosuccinate or aerosol OT
MAPS   3-(trimethoxysilyl)propyl methacrylate
NE   Natural Celluclast BG enzyme complex (Novozymes)
PE   Pretreated Celluclast BG enzyme complex

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SEN   Single enzyme nanoparticle
TEM   Transmission electron microscope

Introduction

The enzymatic degradation of cellulose or lignocellulosic biomass to glucose under industrial conditions is a crucial point in industrial bioethanol production [1]. The utilization of the lignocellulosic biomass is important to get more renewable sources for biofuel production [2]. These techniques are intensively investigated worldwidely to elaborate an economic technology for it [3]. The enzyme stabilization techniques could essentially contribute to this goal. Cellulose is a very stable, renewable natural polymer that is present in all plants and algae. Cellulose is derived from D-glucose units, which condense through β(1→4)-glycosidic bonds [4]. Cellulase enzyme complex comprises of three types of enzymes: endoglucanase, which cleave internal β-1,4-glucosidic bonds; exoglucanase, which processively acts on the reducing and non-reducing ends of cellulose chains to release short-chain cello-oligosaccharides; and β-glucosidase, which hydrolyze soluble cello-oligosaccharides (e.g., cellobiose) to glucose. The cooperative action of the three enzymes is required to achieve efficient enzymatic hydrolysis of lignocellulosic biomass [1–3, 5]. This complex is a functional unit, where different enzymes work at a strict molar ratio. There is often covalent interaction between the enzyme molecules of cellulase enzyme complexes producing cellulosome [6].

Celluclast BG enzyme complex, extracted from *Trichoderma reesei*, investigated in both its native and pretreated forms in this work, does not build up as cellulosome, but the enzymes are working synergetically during the cellulose degradation. Each individual enzyme has a well-defined spatial order [7]; this self-arranged supramolecular unit is one type of hyperstructure [8]. A hyperstructure is a large, spatial association of cellular constituents (e.g., enzymes) that performs a particular function because the constituents are associated with one another [8].

Improvement in enzyme stability is an important goal of practical applications of enzymes for digestion of the lignocellulosic biomass. Classical techniques for improving the stability of the enzymes are enzyme immobilization to the surface or inner cavity of a carrier, modification of the surface of the enzyme, protein engineering, reaction medium engineering, and cross-linked enzyme crystals [9]. Recently, growing interest is being seen in using nanoparticles as carriers to achieve enzyme immobilization in order to increase enzyme stability. Two main routes [A) and B)] can be distinguished in synthesizing nano-sized enzyme carriers [10]. The route A, the so-called grafting onto method, involves two main steps. The first one is the synthesis of a nanocarrier material (Fig. 1a). Wide range of particles can serve as a carrier, such as silica nanoparticle [11], metal nanoparticle [12], or different types of magnetic nanoparticles [13–21], mesoporous materials such as mesoporous organosilica [22], hollowed metal or silica nano-sphere [23, 24], magnetic mesoporous carbon [25], hyperbranched polymer [26], or dendrimer [27, 28]. As second step, enzyme molecules are attached to the surfaces or onto the inner cavities of the carrier materials. The route B, the "grafting from" modification, means in situ growth of carrier material from the surface of the enzyme molecules [10]. Nanostructure formation and attachment of this structure to the enzyme are integrated into a single step (Fig. 1b). The nanostructure can be an inorganic layer (e.g., mesoporous silica [29], magnetic nanolayer [30]), or polymer gel (organic [31] or an organic/inorganic hybrid polymer [32, 33]; Fig. 1b).

Single enzyme nanoparticles (abbreviated as PE, pretreated enzyme) applied in this work are prepared by the "grafting from" method [32–34]. Single enzyme nanoparticles means
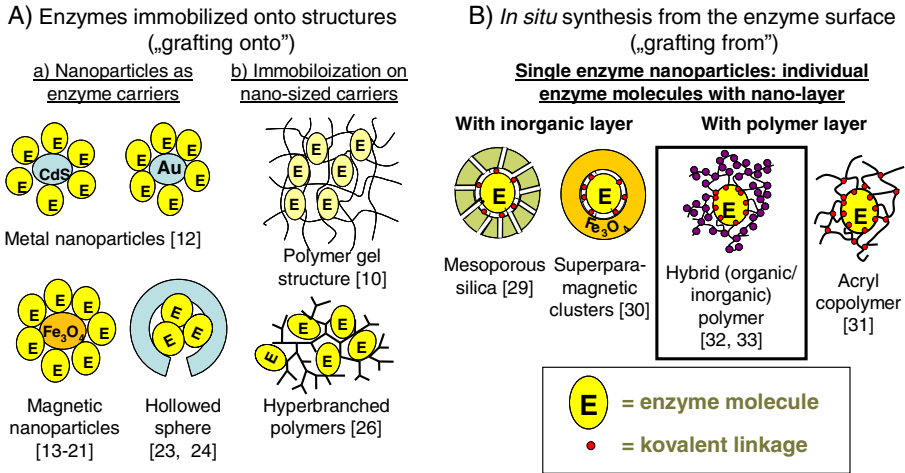
Fig. 1 Nanotechnological methods for enzyme stabilization. The new approach is the reduction of the size of the enzyme carriers using (1) *a* metal or *b* magnetic nanoparticles as carriers, (2) encapsulation into *a* hyperbranched polymers and *b* dendrimers. (3) Single enzyme nanoparticles means single enzyme molecules encapsulated with **a** polymer network [α) organic/inorganic hybrid polymer or β) nanogel] **b** inorganic layer [(α) hollow metal sphere, (β) mesoporous silica, or (γ) magnetic nanolayer]

encapsulation of single enzyme molecules within a few nanometers thick layer [32] which results in the stabilization of enzyme activity without any serious limitation of the substrate transport from solution to the active site of the enzyme [33, 34]. The synthesis of PE is available via a more or less simple laboratorial technique [32–34]. This technique was previously applied on the chymotrypsin enzyme only, and the activity of SEN enzymes was investigated using relatively small substrate molecules as *N*-acetyl-L-tyrosine ethyl ester [32, 33]. It was not clear that the covered enzyme nanoparticles can digest large substrate molecules, for example cellulose macromolecules, which is crucially important for hydrolysis of cellulosic biomass in order to produce biofuel. The total cellulase activity of the pretreated and the native enzymes has been measured in order to get how strongly the stability of the enzyme can be improved by its pretreatment. For it, the hydrolysis of Whatman type filter paper was used. This method was applied since 1984, when the Commission on Biotechnology of the International Union of Pure and Applied Chemistry proposed a number of standard procedures for the measurement of cellulase activity [35]. That is why this technique has been applied to measure the activity of the cellulase enzyme complex, and cellulose filter paper has been used as a substrate (Whatman type filter paper). The main aim of this work is to prove that the pretreatment method used can serve enzyme nanoparticles with much longer stability than the native one applying it for digestion of the large polysaccharide molecules.

Materials and Methods

For the preparation of enzyme nanoparticles using cellulase enzyme complex, the following chemical compounds were used: Celluclast BG enzyme complex from *T. reesei* (Novozymes), acryloyl chloride, 1,3-bis[tris(hydroxymethyl)methylamino]propane or Bis-Tris propane, 3,5-dinitro-salicylic acid (Sigma), sodium bis(2-ethylhexyl) sulphosuccinate or

aerosol OT (AOT), methacryloxypropyltrimetoxysilane (MAPS), 2,2-azobis(2,4-dimethyl-valeronitrile; Fluka), disodium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, 2-propanol, and n-hexane (Spektrum-3d, Scharlau).

Julaba F12 cryostat was used to keep the reaction mixture at 0 °C. The polymerization step in the synthesis of single enzyme nanoparticles of Celluclast BG enzyme (PE) was carried out in a double-walled stirred vessel (Fig. 2). The solution was irradiated by an UV lamp made by Vilber Lourmat. Filtration of the surface-polymerized enzymes was carried out with a syringe filter (pore size, 0.1 μm) made by Millipore. UV spectra were recorded and enzyme activity measurements carried out by means of a Biochrom 4060 spectrophotometer made by Pharmacia. A New Brunswick Scientific G24 incubator shaker was used for the stability measurements. Malvern Zetasizer (Nano series Nano-ZS, Malvern Instruments Ltd. Worcestershire, England) was applied for detection of size distribution of the enzyme nanoparticles.

Preparation of Cellulase Enzyme Nanoparticles

The preparation process of cellulase enzyme nanoparticles (PE) has three steps (Fig. 3). The detailed description of the procedure was given earlier [32–34, 36].

1. The first step is a modification of Celluclast BG enzyme complex (native enzyme, NE) and its solution in a hydrophobic medium following the method of Wang et al. [37] (1) Primary amino groups on the surface of the enzyme are modified by acryloyl chloride. One hundred milligrams of NE was dissolved in 50 ml distilled water. Forty microliters of acryloyl chloride was added to the solution under 0 °C (the unreacted acryloyl chloride was removed using dialysis membrane with cutoff 10 kDa after half an hour of reaction time). Then, the solution was mixed with 50 ml double concentrated stock solution. The resulting solution contained 1 mg/ml acryloylated NE, 1 % (v/v) isopropanol, and 2 mM $CaCl_2$ dissolved in 10 mM Bis-Tris buffer at pH=7.0. There was no measurable decrease in enzyme activity after the modification of NE with acryloyl

Fig. 2 UV irradiation for the polymerization on the surface of the single enzyme molecules
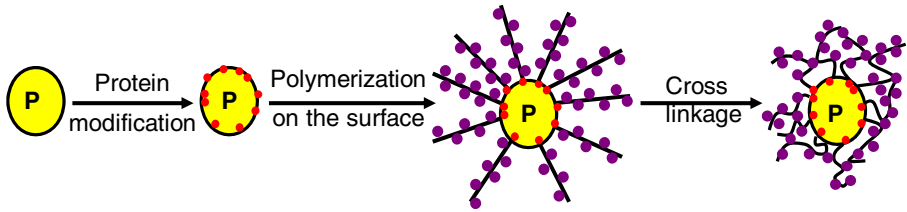
Side view
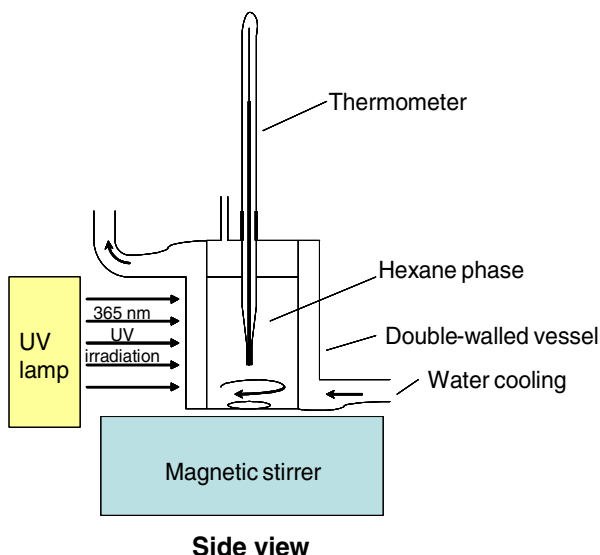
Fig. 3 Synthesis steps for preparation of single enzyme nanoparticles

chloride. (2) the modified NE was dissolved in hexane media using the "hydrophobic ion pairing" method [38, 39]. Fifty milliliters of an aqueous enzyme solution was added to an equal volume of hexane containing 4 mM AOT surfactant. The resulting two-phase mixture was stirred and centrifuged at $7{,}000 \times g$ at room temperature for 10 min.

2. After that, the solution of the acryloylated NE in hexane, 3-(trimethoxysilyl)propyl methacrylate (MAPS, 1,325 μl) was added to 10 mg acryloylated NE in 50 ml hexane. Free radical polymerization was initiated between the vinyl groups of MAPS monomers and acryloylated NE under UV irradiation (365 nm), in the presence of 0.8 mg/ml 2,2′-azobis-(2,4-dimethylvaleronitrile) as initiator. This polymerization step was performed under UV light in a double-walled and water-cooled glass vessel, for 8 h at room temperature (UV light of this wavelength is not absorbed by the glass wall).

3. The final (third) step was the hydrolysis and condensation of the trimethoxysilyl functional group. This process was carried out in an aqueous phase. For this reason, an equal volume of aqueous phosphate buffer (10 mM phosphate buffer, pH=7.8) was added to the hexane phase. The resulting two-phase system was stirred with a magnetic stirrer at 300 rpm at 22 °C for 5 min. The aqueous buffer phase was filtered using a syringe filter unit (maximum pore size, 0.1 μm).

Characterization of the Enzyme Nanoparticles

The morphology and the size of the resulting PE were examined by a JEOL-1200X transmission electron microscope (TEM) at an accelerated voltage of 80 kV. The procedure of the imaging was rather simple; the sample did not need specific preparation for the measurement. One drop of the PE solution was enough to get detectable image by the TEM. The drops of PE samples were dried, and after the drying, the enzyme nanoparticles were detected.

Measurement and Calculation of Enzyme Concentration

The concentration of the natural Celluclast BG enzyme complex (NE) was determined by taking calibration measurements of the enzyme absorption at 280 nm. There was no found difference between the absorption properties of modified and native enzymes. But the concentration of the pretreated Celluclast BG enzyme complex (PE) could not be obtained from the measurement of the UV light absorption because the polymer layer prepared around the enzymes also has got high absorption at wavelength 280 nm. The PE concentration in water was therefore calculated from the initial concentration of modified NE in hexane (in this case the absorption was measurable), assuming that after the polymerization step the full amount of PE was transferred from the hexane into the water phase, and the amount of PE precipitated during the phase transfer process was negligible.

Measurement of Enzyme Activity

Whatman filter paper was used as substrate (filter paper unit, FPU), and total cellulase activity was measured using DNS probe according the instructions of Ghose [35, 40]. One milliliter of 0.05 M citrat buffer (pH=4.8) and 0.5 ml of the cellulase (NE or PE) stick solution were mixed, and filter paper sample was added into the mixture as a substrate (1× 6 cm Whatman no. 1. standard filter paper), and the mixture was incubated at 50 °C for 60 min without shaking. After the incubation, the enzyme–substrate mixture was cooled, and the next steps were the standard activity measurement (DNS probe was used to measure the total cellulase activity after the treatment) [40].

Measurement of Enzyme Stability

The heat stability of native and pretreated enzymes was measured at the following method: the stick solutions were incubated at the desired temperatures (from 37 to 80 °C). The incubated stock solutions (0.5 ml) of NE and PE were sampled to the activity measurement (see above). Three samples were measured, and mean values were calculated. The relative activity was calculated as the ratio of residual activity to the initial activity.

The pH stability was measured similarly to the activity measurements: using 1 h incubation under different pH values at 50 °C. Whatman filter paper was used as substrate (FPU).

Results and Discussion

The size distribution, structural characteristics, and activity of the pretreated enzyme nanoparticles (PE) were investigated. Activity of PE product was also measured by incubating it under different, sometimes even extreme, conditions. These results are briefly summarized in the next subsections.

Size Distribution of PE During the Preparation Process

The aim of the pretreatment is to form single enzyme nanoparticles where the single enzymes are covered by thin polymer layer. The size distribution gives information on the size of these aggregations as well as on what portion of enzyme forms aggregations. If the average size of the resulting PE product is in the same range as the original size of the NE, one can conclude that there is no detectable aggregation in the PE product. Otherwise, one can detect the amount of the aggregated enzymes during each step of the synthesis process. The size distribution of the PE was measured by Zetasizer after the preparation. The size distribution of the initial native cellulose enzyme complexes (NE in aqueous solution, blue line), the surface-modified enzymes dissolved in hexane (NE in hexane solution, green line), and pretreated enzymes (PE in aqueous solution, black line) are illustrated in Fig. 4. The average particle size of native Celluclast BG enzyme complex is about 4 nm, and that of modified NE dissolved in hexane by ion pair is 9 nm, while that of PE product in water is about 11 nm. That could mean that the thickness of the polymer layer formed around the enzyme is about 3 nm.

Structural Characterization of Pretreated Enzymes

Transmission electron microscopic images can prove that the polymer layer is really formed around the enzyme molecules. Figure 5 shows that the resulting enzyme nanoparticles have
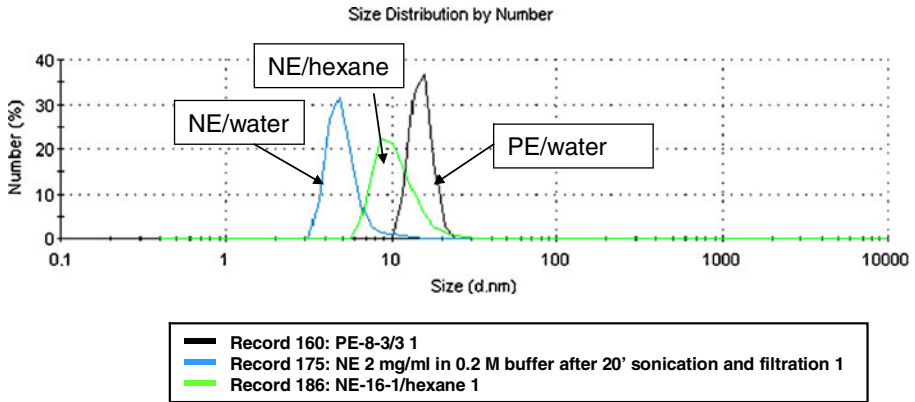
Fig. 4 Size distribution of aqueous solution of the native cellulase enzyme complex (NE, *blue line*), surface-modified cellulase enzymes dissolved in hexane (NE, *green line*), and pretreated cellulase enzymes dissolved in water (PE, *black line*)

hollow spherical structures on the electron microscopic images. The surrounding silica-containing nano-layer is electrodense and results in a dark layer around the enzyme molecules on the picture.

The size of the PE particles is about 5–30 nm (Fig. 5). The results obtained confirm that this technique is suitable to realize the enzyme complex nanoparticles making smaller polymer layer around single enzyme molecules of the enzyme complex which are separable (smaller particles in Fig. 5), or in some cases, a few enzymes can be included in the nanoparticles (greater particles in Fig. 5). (Larger particles on the TEM image could be produced by agglomeration of nanoparticles during the preparation of the sample to these measurements). The thickness of the polymer layer can be estimated to be about 3 nm according to Fig. 5. The cellulase enzyme complex contains three different types of enzymes with a different function. The diameter of cellulosome in the case of *Clostridium thermocellum* is about 18 nm [41]. Celluclast BG enzymes cannot make cellulosome (there is no covalent binding between the enzymes), but the precise spatial arrangement of enzymes during their cooperative cellulose digestion is critical for the good function.

Activity of Enzyme Complexes Using Native Substrates

The question to be answered is whether single enzyme nanoparticles can degrade large substrate molecules as polysaccharides, as well. It can be imaged that the polymer layer

Fig. 5 TEM images of the pretreated Celluclast BG enzyme complexes (PE)
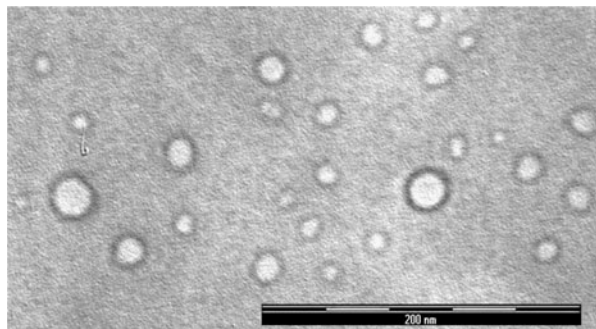
around the enzyme molecules is not porous enough to allow the free diffusion of larger substrate molecules from the solution to the active center of the enzyme. The activity of the enzyme nanoparticles indicates whether the substrate molecules can connect to the active site of the enzyme or not. Natural crystalline cellulose polymer was used as substrate. Most cellulases have a catalytic domain (the active site of the enzyme) and a cellulose binding domain that anchors the enzyme onto the cellulose surface and orients the cellulose fiber towards the tunnel containing the active site [42, 43]. Total activity of enzyme complex was measured both of NE and PE. Turnover number or molar activity of cellulase enzyme complex can not be calculated because the activity of cellulase enzyme complex is determined by different enzymes with a different molecular weight. For this reason absolute activity of celluclast BG enzyme complex was expressed in units/mg. The absolute value of the activity of natural Celluclast BG enzyme complex (NE) was 2.84 Unit/mg. In the case of the pretreated enzyme complex (PE) the absolute value of the activity was 0.746 Unit/mg. (This value was applied to a pure enzyme mass without the weight of the polymer layer around the enzyme molecules.) The absolute value of the activity of PE has 26.27 % of the activity of NE which is acceptable in practical points of view. It can be stated that enzyme nanoparticles of Celloclast BG enzyme complexes with a polymer layer (PE) can degrade as great and stable substrates as native crystalline cellulose polymer. That should mean that the polymer layer around the enzyme molecules is thin, porous, and flexible enough to enable the interaction between the cellulose binding sites of the cellulase enzymes inside the polymer layer and the cellulose polymer outside the polymer layer of PE. Thus, this layer does not hinder the formation of enzyme–substrate complex and does not make practically diffusive limitation to it. It means that the polymer layer does not limit the function of domains of cellulase enzymes.

Temperature Stability of Pretreated Enzymes Under Different Conditions

The pretreatment of an enzyme can serve to form a much more stable enzyme than the native one. The PE (continuous line in Fig. 6) retains about 40 % of its initial activity value after a 110-day period, while the NE (not pretreated, dotted line) Celluclast has lost its activity after 11 days at room temperature (20 °C). The results show that the stability of the PE is at least one order of magnitude better than that of the NE. After the first short and quickly decreasing period of the activity of PE, the gradient of the stability's decrease of the PE is

Fig. 6 The relative activity (activity at different times related to that of the starting time) of PE and of the NE at 20 °C without stirring (*X* native cellulase enzymes, *O* pretreated cellulase enzymes)
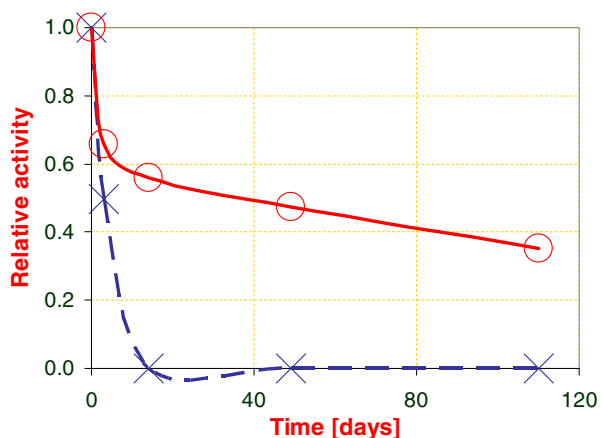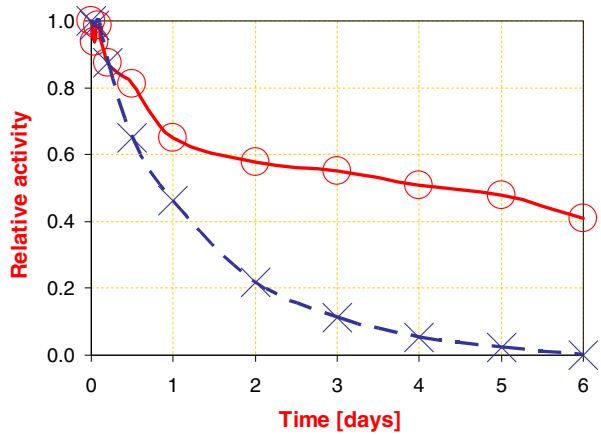
Fig. 7 Stability of PE and NE at 37 °C and stirring speed of 150 rpm (*X* native cellulase enzymes, *O* pretreated cellulase enzymes)

much lower than that of the NE (Fig. 6). The NE loses its activity within about 15 days, while the pretreated one retains more than 50 % of its original activity. This activity lowers slowly during the 120 days investigated. The prepared enzyme keeps about 35 % of its starting activity even after this long period of time.

Similarly, the activity of PE is higher than that of the NE incubating them at 37 °C under stirring conditions with 150 rpm. The NE loses its activity during 6 days under the above conditions, but about 40 % of its starting activity of PE remains during a 100-day incubation time (Fig. 7). The pretreated enzyme gradually also loses its activity as a function of time, but this decrease is much slower than that of the native one.

At extreme temperature, namely 80 °C, and without stirring, the activity of the PE is also much higher than that of the native one. The NE loses its whole activity after 6 h of incubation time, while the PE retains about 40 % of its original activity after a 12-h incubation time (Fig. 8).

The activity change of the PE and NE during 1-h incubation time is plotted in Fig. 9, measured at different temperatures between at 50 and 80 °C. The procedure of these measurements is given in the "Measurement of Enzyme Activity" section. There is no difference between the activity of NE and PE at 50 °C for 1-h incubation, but increasing

Fig. 8 Stability of PE and NE at 80 °C (*X* native cellulase enzymes, *O* pretreated cellulase enzymes)
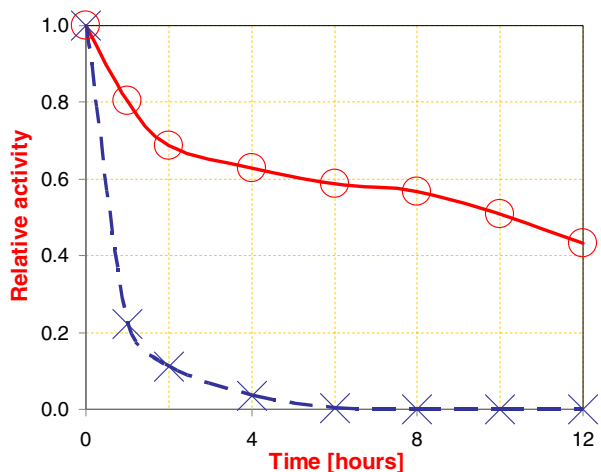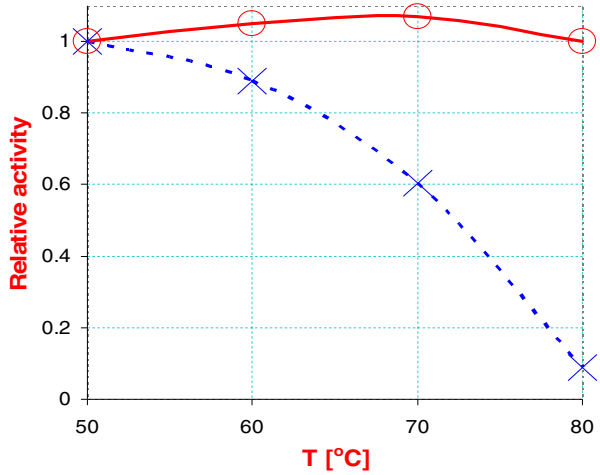
Fig. 9 Activities of PE and NE at difference temperatures (*X* native cellulase enzymes, *O* pretreated cellulase enzymes)

the temperatures, the activity of NE strongly lowers, while the activity of PE does not change practically. The activity of NE is decreased down to about 10 % of its activity at 80 °C, while that of PE remains practically at its starting value.
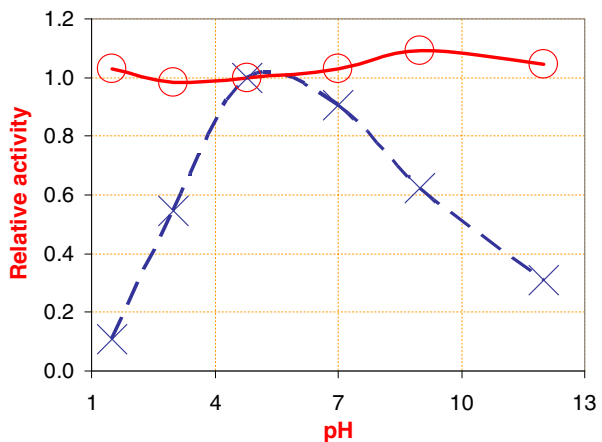
pH Stability of Pretreated Enzyme Complex

The PE retains its activity not only at extremely high temperatures but also at an extremely wide pH range. The activity of the NE enzyme complex decreases below 15–25 % of its activity measured at pH=6.0, in the pH range of 1.5 and 12.0, while the activity of the pretreated enzyme PE changes only slightly (Fig. 10). Further investigations are needed to clarify what is the reason for this surprising property.

Conclusion

The Celluclast BG enzyme complex has been covered by a few nanometers thick polymer layer in order to improve its stability. This layer isolates the enzymes from the environment

Fig. 10 pH stability of PE and NE (*X* native cellulase enzymes, *O* pretreated cellulase enzymes)

which can act positively on its stability. It has been proved that the pretreated enzymes could be much more stable than the native, not pretreated enzymes even in extreme temperatures and pH environment. The covered Celluclast BG enzyme complex (PE) retains its activity at least ten times longer than that of the native one, at a temperature range between 20 and 37 °C. The pretreated enzyme complex can hold back about 50 % of its original activity after 12 h of incubation time at even 80 °C, while the native Celluclast BG enzyme complex (NE) totally loses it during 6 h of incubation. The activity of PE has not been reduced significantly even at extreme pH values as 1.5 and 12.0. Consequently, the pretreatment method can essentially widen the industrial application of the enzyme-catalyzed bioreaction under more extreme environmental conditions, as well.

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