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CELLULASE AND HEMICELLULASE ENZYMES AS SINGLE MOLECULAR NANOBIOCOMPOSITES

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We have worked out two different stabilization procedures for glycolytic enzymes with significant biotechnology relevance: for the commercial Novozyme cellulase complex (Celluclast) and for recombinant *Thermobifida fusca* hemicellulases (β-D-xylosidase and β-D-mannosidase). In the applied methods, individual cellulase and hemicellulase enzyme molecules were conjugated with a polymer nano-layer providing nanobiocomposites (single enzyme nanoparticles, SENs) that exihibit an excellent stability under extreme conditions. The first method that we have been successfully used earlier on chymotrypsin [3], creates a polymer layer around the enzyme molecules with trimethoxysilyl (TMS) functionalities in three steps [4]. The second stabilization method is a novel one pot reaction resulting in an acrylamide-bisacrylamide (AA) copolymer layer around the enzyme molecules in two steps. The heat stability of the developed single enzyme nanoparticles with TMS and AA nanolayers were tested at two different temperatures (+4 °C; 80 °C) following the residual activities of the modified enzymes in regular time interval. Upon incubation of the cellulase complex nanoparticles with TMS and the non-modified cellulase at 80 °C, the nanoparticle has kept its 40% of its starting cellulase activity after 6 hours, whereas the non-modified enzyme lost its activity completely in half an hour. The relative activity of SENs with AA is about 50% of the initial activity. After 12 hours under 80 °C the activity of NCK is reduced to 14%, while the activity of NCK_A is 24% of the activity of the native enzyme at the start of the incubation. The nanoparticles of the *Thermobifida fusca* hemicellulases (β-D-xylosidase and β-D-mannosidase) obtained from both TMS and AA nanolayers have exhibited concomitant stability increase. TMS nanoparticles of β-xylosidase has lost 60% of its activity after 120 days during an incubation procedure at +4 °C, whereas the non-modified β-xylosidase lost all its activity in 40 days during the same conditions. The 40% of the starting enzyme activity of β-D mannosidase with AA has remained after 6 hours, during an incubation process at 80 °C, the activity of the non-modified enzyme has lost in one hour.

Keywords: thermostable enzymes, single enzyme nanoparticles, hemicellulases, *Thermobifida fusca* species.

Introduction

Extremophilic organisms require physically or geochemically extreme conditions that are harmful for most other organisms on Earth. Thermophilic organisms are a type of extremophile organisms that lives at relatively high temperatures, between 45 °C and 80 °C. Cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also thermostable. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications [1].

Thermobifida spp. are gram-positive, compost- and soil-inhabiting bacteria with broad decomposing activity on plant cell wall constituents. *Thermobifida fusca*, the most extensively studied species of this genus, is the model organism of thermophilic, aerobic cellulolytic bacteria. *Thermobifida fusca* produces multiple extracellular enzymes including cellulases that are responsible for the decomposition of cellulose and lignocellulose residues, which make up the bulk of agricultural and urban wastes *(Fig. 1)*. While there are several data on the cellulolytic system of *T. fusca*, the hemicellulolytic enzyme system of this species is still poorly characterized [1]. Hemicelluloses act as linkers between lignin and cellulose. The high percentage of hemicellulose fraction in the cell wall of higher plants makes this material the second most abundant biopolymer in nature. Single enzyme nanoparticles (SENs) represent a new approach in industrial enzyme research [2, 3] *(Fig. 2)*.

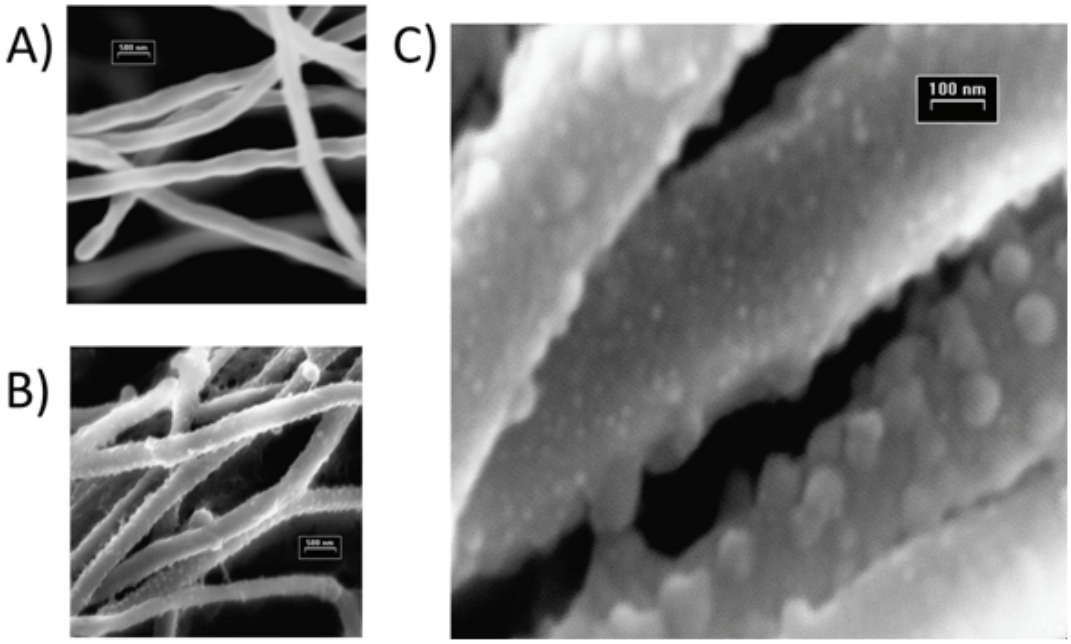

Figure 1: Hyphal surface of *Thermobifida fusca* TM51
A) Smooth surface of mycelium grown on glucose
B) Cellulosome-like structures emerged upon induction by cellulose
C) Cellulosome-like structures at higher magnification

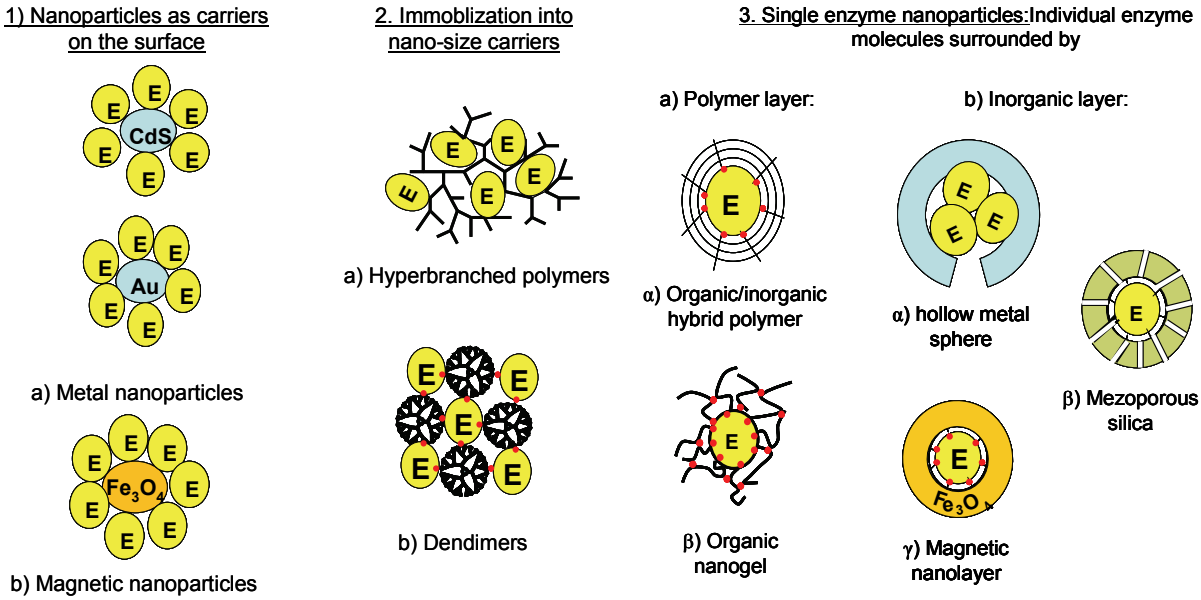

Figure 2: Nanotechnological methods for enzyme stabilization

The form of SEN means, that each enzyme molecule are surrounded with a nanometer scale polymer matrix layer, resulting in stabilization of enzyme activity without any serious limitation for the substrate transfer from solution to the active site of the enzyme. The synthesis of SEN particles is available via more or less simple laboratorial technique. Previously we have decided to apply this technique for industrial bioethanol synthesis able to comply with the requirements of green chemistry. We would like to investigate that how can the SEN-enzymes digest higher-size substrates.

Single enzyme nanoparticles can be used for stabilization of enzymes in industrial enzyme research. Using mesoporous silica gels single enzyme nanoparticles can be fixed into the inner wall of the mezoporous gel and it means that nanoreactors can be fabricated [2]. The polymer layer of the single enzyme nanoparticles is electrodense to transmission electron microscopic detection the structures of the enzyme complexes can be realized easily. Polymers composed with MAPS monomers have a good biocompatibility and has no toxicity [14, 16]. We investigated the chemical stability of different enzymes and enzyme complexes in the form of SEN. Otherwise a numerous types of enzymes and proteins (lipases, bovine serum albumin and hemicellulases from *Thermobifida fusca*) can not be solved in hexane. Recently a new technique was found, which does not need any specific solution

technique in hexane because the polymerization of the nano-layer around the enzyme molecules can be realized in water media. For the polymerization we used a mixture of mono and bifunctional monomers (acrylamide and bisacrylamide mix), the preparation of enzyme nanoparticles can reduced to one-pot reaction with two steps [4].

Materials and methods

Enzymes

Novozyme Celluclast BG cellulase enzyme complex (CK) and *Thermobifida fusca* wild type endomannanase (EM), β-xylosidase (XI), β-mannosidase (MN) and mutant β-mannosidase (MM) glycolytic enzymes were used in these experiments. Hemicellulase enzymes were hererologously expressed as His-fusion protein constructs in *Escherichis coli* BLD21 host and were further purified by affinity chromatography on NiNTA-agarose beads and isolated as a 95% homogeneous, highly active enzyme sample by József Kukolya (Szent István University, Faculty of Agriculture and Environmental Sciences) and Terez Barna and Csaba Attila Fekete (University of Debrecen; Department of Genetics and Applied Microbiology).

Other chemicals

Acryloyl chloride, 1,3-bis[tris(hydroxymethyl)-methyl-amino]propane or Bis-Tris propane (Sigma), sodium bis(2-ethylhexyl) sulphosuccinate or aerosol OT (AOT) (Fluka), disodium hydrogen phosphate, potassium dihydrogen phosphate, calcium chloride, 2-propanol, n-hexane (Spektrum-3d, Scharlau), methacryloxypropyl-trimetoxysilane (TMS), 2,2-azobis(2,4-dimethylvalero-nitrile) (Fluka), 3,5-dinitro-salicylic acid (Sigma), acrylamide (Sigma), bisacrylamide (Sigma), tetraethyl-orthosylicate (TEOS, Sigma), ammoniumperoxo-disulphate (Sigma), paranitrophenyle-β-D-manno-pyranoside (Sigma), paranitrophenyle-β-D-xylopyranoside (Sigma).

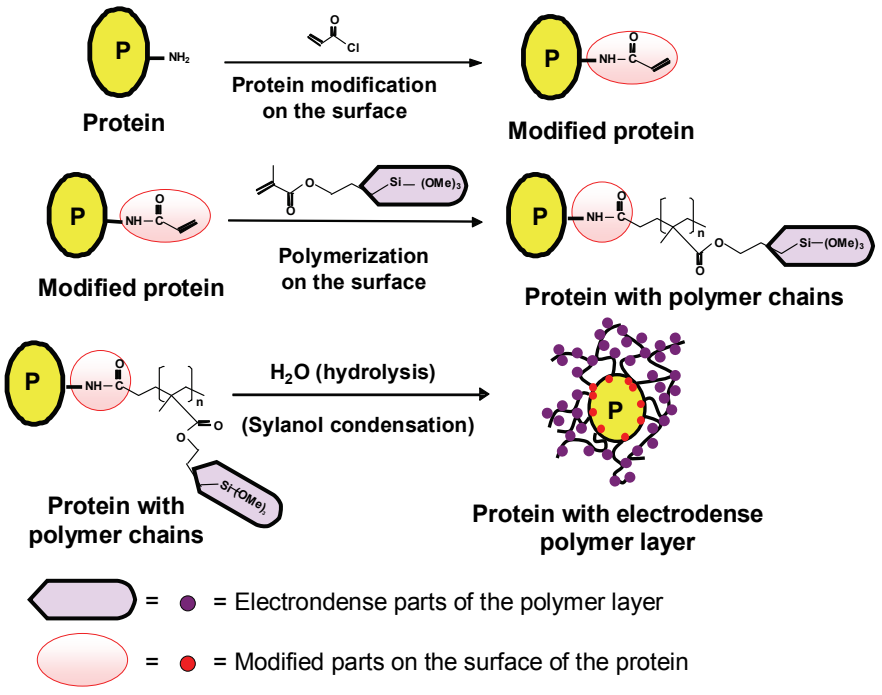

Figure 3: Thee-step method to synthesize single enzyme nanoparticles

Instruments

Cryostate was used to keep the reaction mixture at 0 °C. A gas chromatographic syringe (volume 5 µl) was used for the addition of a few microlitres of acryloyl chloride to the enzyme solution. The polymerization step in the synthesis of single enzyme nanoparticles (SEN) was carried out in a double-walled stirring vessel. The solution was irradiated by a 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. For the detection of the size distribution of the enzyme nanoparticles Malvern Zeta-sizer was used.

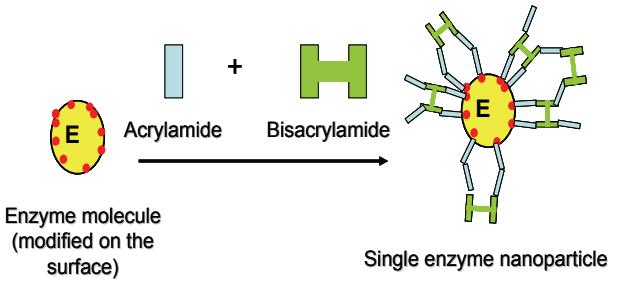

Figure 4: One-pot reaction to synthesize single enzyme nanoparticles

Methods

Synthesis of SEN: We used two methods for the preparation of single enzyme nanoparticles (SEN). The first method has three steps *(Fig. 3)*. The description of the procedure was detailed earlier [2, 3]. The first step is a modification of CK enzyme complex and its solubilization in a hydrophobic medium. The second step is the polymerization of the vinyl group in hexane, and the final (third) step is hydrolysis and condensation of the trimethoxysilyl functional group (TMS). For the polymerization step, the enzyme should be dissolved in a hydrophobic medium (n-hexane). The surfaces of the enzymes have hydrophobic and hydrophilic regions. If hydrophilic parts of the surface of the enzymes are covered by surfactant molecules, the enzyme lost its hydrophilic characteristic and became hydrophobic, so hydrophobic solvents, e.g. hexane can solve it easily. During this complexation ion-pairs make bounds between the ionic part of the surfactant and the ionic part on the surface of the enzyme. For the polymerization on the surface of the enzyme direct contact is necessary between the enzyme surface and the hexane medium (Fig. 3). In this process, vinyl groups on the enzyme surface (synthesized in the first step) are well exposed to the organic solvent (and reagents). Preparation of single enzyme nanoparticles can be realized also using a one-pot reaction *(Fig. 4)* [4]. This method has two steps. The first step is the modification of the surface of the enzymes (this step is similar in both method). The second step is a polymerization in water media using acrylamide and bisacrylamide substrates as mixtures (9: 1 molar ratio of acrylamide: bisacrylamide).

Measurement of enzyme activities: In the case of cellulase enzymes Whatman filter paper was used as substrate (filter paper unit, FPU) and total cellulase activity was measured using DNS-probe [17]. Activities of hemicellulase enzymes was measured using chemically modified substrates. The activity of β-D-mannosidase was measured using paranitrophenyle-β-D-manno-pyranoside as substrate. Activity was calculated by the absorbance changes at 400 nm. Activity of β-D-xylosidase was measured and calculated in similar way using paranitrophenyle-β-D-xylopyranoside as substrate. (Enzyme concentrations were calculated by absorbance at 280 nm using molar extinction coefficients.)

Results and Discussion

Two methods were used to synthesize enzyme nanoparticles. The first method (by Kim at al., [2, 3]) contains three steps and the second method (by Yan et al., [4]) is a one-pot reaction. Enzyme nanoparticles using β-mannosidase and cellulase enzymes were created by both methods and therefore we can compare the yields of the enzyme nanoparticles using these methods.

Effectivity of methods

During the synthesis steps of the Kim method, the yields of the products at the end of each step could be less and less than the initial amount of enzymes before the stabilization processes. Hemicellulase enzymes from *Thermobifida fusca* species precipitated at the first step because these enzymes are very sensitive for pH-changes. Therefore the yields of the enzyme nanoparticle products from these hemicellulase enzymes are very low, less than 5 percent (except β-xylosidase enzyme, where the yield of enzyme nanoparicle is about 12%, *Table 1*).

But even in the case of cellulose enzyme complex from *T. reesei* (Celluclast BG from Novozymes), where each step has resulted in a relatively good yield (more, than 60%), at the end of the whole process the yield became only 15–25% (*Table 2*, first column).

The second method (by Yan et al.) does not contain phase transfer or solution change steps [4], therefore much higher yield could be achieved at the end of this stabilization process than that of the Kim's method (Table 2).

Table 1: Yields of enzyme nanoparticles of thermostable hemocellulase enzymes (from *Thermobifida fusca*) using method by Kim et al.

Type of enzymes	**β-mannosidase**	**Mutant β-mannosidase**	**β-xylosidase**	**Endomannanase**
Sign	MN–12	MM–2	XI–6	EM–4
Amount [mg] and yield (η) of enzyme nanoparticles	0.525 (η = 3.50%)	0.782 (η = 4.01%)	3.712 (η = 12.37%)	0.280 (η = 4.15%)

Table 2: Comparison of the yields of enzyme nanoparticles using two methods

Methods		Kim et al. (3 step process)	Yan et al. (one pot reaction)
Yields [%]	**β-mannosidase**	7%*70%*60%= **=3%**	**21%**
	Cellulase enzyme complex	60%*70%*60%= **=15-25%**	**47%**

Size distribution

We have examined and followed the size distribution of endomannase nanoparticles with AA monolyers and TMS monolayers. The peak of the size distribution of the non modified endomannanase enzyme (EM/water) was about 6 nm. There was no difference between the size of the non-modified EM enzymes and the surface-modified acrylated EM enzymes. In contrast, using the three step Kim method, the size distribution of the endomannanase enzyme nanoparticles were varied during the preparation using the Kim's method. The range of the size distribution of the EM nanoparticles after the hydrophobic ion pairing process measured in the hexane phase are between 5–11 nm (EM/hexane, *Fig. 5A*). After the third step (polymerization with TMS) there was a further increase in the size and the peak of the size distribution of the SEN-endomannanase (SEN–EM/water) is about 15 nm (Fig. 5A). In *Fig. 5B* we compared the size distributions of SEN-products in the case of three different enzymes (EM = endomannanase, MM = mutant mannosidase, MN = β-mannosidase, XI = β-xylosidase enzyme, Fig. 5B). SEN–EM and SEN–MM products have about same size but SEN–MN and SEN–XI have higher peak of their size distribution (about 60 nm).

For a good enzymatic function of β-xylosidase enzymes has a quaternary structure it is a homotetramer. The monomeric form of β-xylosidase has no enzymatic activity. Results suggest that this technique could preserve the fine interactions between the enzyme molecules and the quaternary structures could remain during the process.

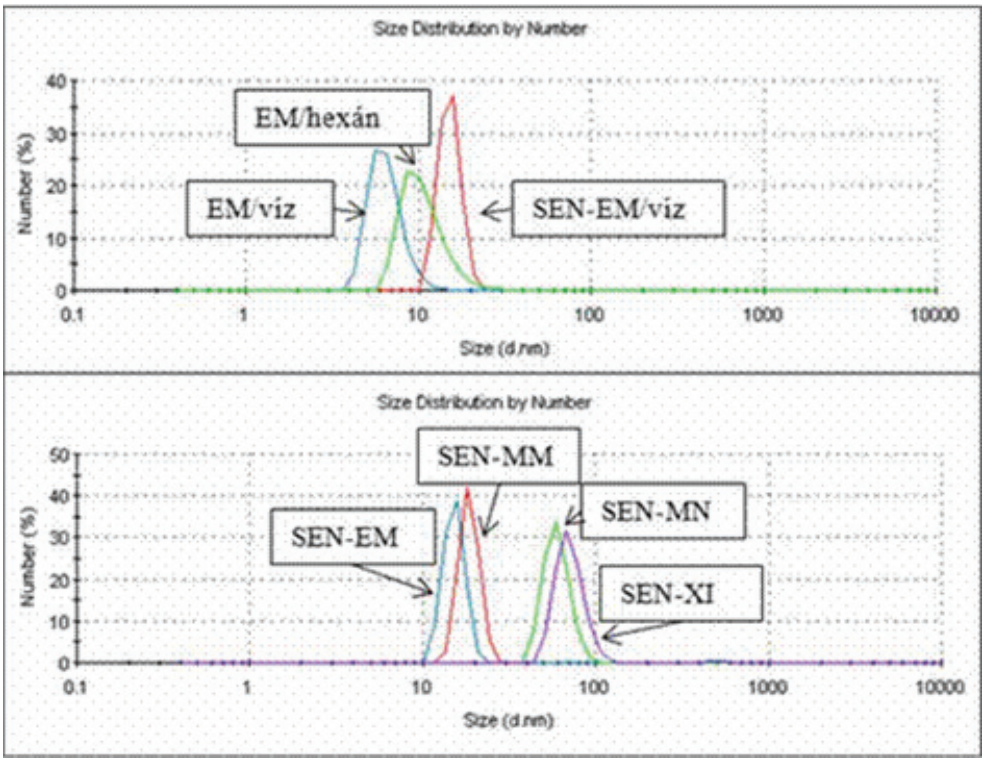

Figure 5: A) Size distribution of endomannanase enzyme (EM) during the three step process of preparation. B) Size distribution of different type of single enzyme nanoparticles after the solution in hexane using hydrophobic ion pairing (EM = endomannanase, MM = mutant mannosidase, MN = β-mannosidase, XI = β-xylosidase enzyme)

Stability measurements

Incubating the single enzyme nanoparticles from β-xylosidase enzyme with TMS at +4 °C for a long period of time, 40% of the enzyme activity has remained after 120 days, while the non-modified β-xylosidase enzyme lost its activity after about 40 days *(Fig. 6)*. Incubation of the enzyme nanoparticles from β-mannosidase with AA at high degree (80 °C), has decreased the mannosidase starting activity to 40% in 6 hours, whereas the non-modified enzyme has lost its activity after a half an hour under the same conditions *(Fig. 7)*. In both cases after a short period of the intensive reduction in the SEN's activity was observed, then there were no further activity loss, and 40% of initial activity was remained.

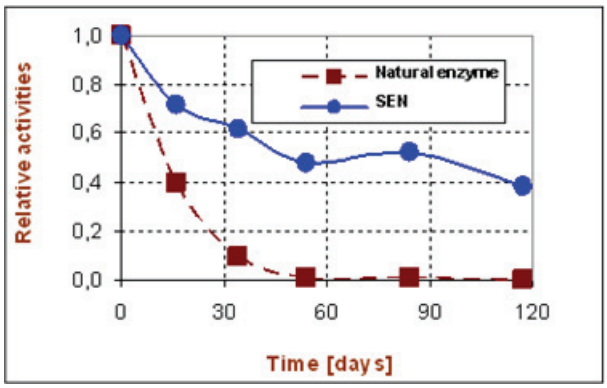

Figure 6: Stability of SEN β-xylosidase enzymes (with TMS) and control β-xylosidase enzymes at +4 °C

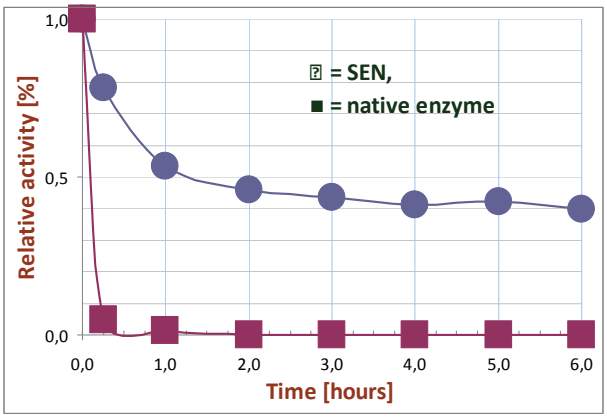

Figure 7: Stability of SEN β-mannosidase enzymes (with AA) and natural β-mannosidase enzymes at 80 °C

Cellulase enzymes (Celluclast BG enzyme complex from Novozymes) nanoparticles were prepared with both methods and the resulted activities and the stabilities of the modified enzymes were compared. NCK is the designation for the enzyme nanoparticles using TMS based nanolayer and NCK_A refers to the enzyme nanoparticles using acrylamide-bisacrylamide mixture (AA) for the polymerization. When double amount of monomers incorporated in the nanolayer, the resulted enzyme nanoparticle is distinguished as NCK_A–1 from NCK_A–2, where the layer contains the usual amount of monomers. We summarize the heat stability of the cellulose nanoparticles obtained by the different methods incubating them at 80 °C, in *Fig. 8*. Both method is able to stabilize the cellulase enzyme complexes at 80 °C. Although the greatest stability increase has obtained in case of NCK_A–2 enzyme nanoparticles when 50% of the initial activity has remained after 12 hours incubation at 80 °C.

We also compared the specific activities of the different cellulose enzyme complex nanoparticles *(Fig. 9)*. The results show that the specific activity of NCK (polymer layer around the enzymes with TMS) is more, than 20% of the non-modified enzyme at the start of the incubation. In the case of NCK_A–2 its activity is 47% of the non-modified CK enzyme at the start of the incubation. After 12 hours incubation at 80 °C, the activity of NCK is reduced to 14%, whereas the activity of NCK_A–2 is 24% of the activity of the non-modified enzyme at the start of the incubation. The initial activity of NCK_A–1 (double amount of monomers in the layer) is about 12% and does not changed during the incubation period. The stability of β-D mannosidase-SEN of *Thermobifida fusca* strain TM51 under 80 °C is also higher than that of the non-modified enzyme. The NMN_A (single enzyme nanoparticles from β-D mannosidase using polymer layer around the enzymes with AA) has about 40% of its original activity, while the native enzyme lost its activity after one hour incubation.

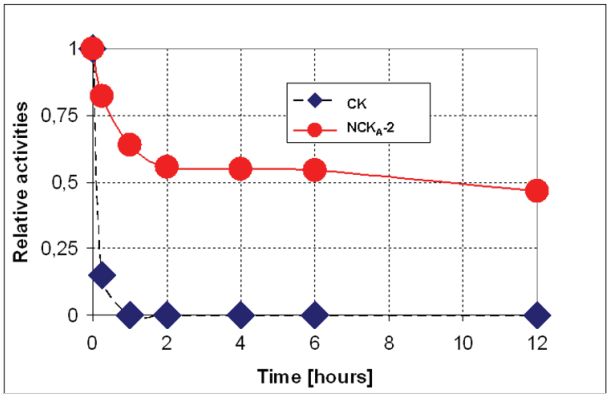

Figure 8: Stability of cellulase enzyme nanoparticles (CK = native cellulase enzyme complex, NCK_A–2 cellulase enzyme nanocomposites with AA at 80 °C)

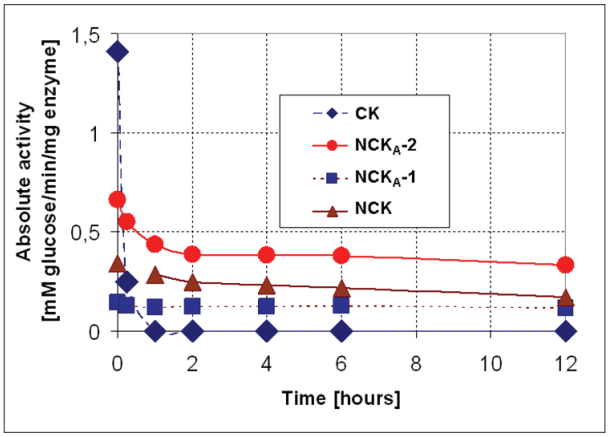

Figure 9: Comparison of the activities of cellulase enzyme complex using different methods to stabilize them as enzyme nanoparticles (CK = natural cellulase enzyme complex, NCK = cellulase enzyme nanoparticles with TMS, NCK_A–1 cellulase enzyme nanoparticles with AA (higher thickness), NCK_A–2 cellulase enzyme nanocomposites with AA (usual thickness) at 80 °C

Conclusions

Cellulase and hemicellulase enzyme nanoparticles were synthesized to stabilize the enzymes under industrial conditions. Thermostable enzymes from *Thermobifida fusca* (β-D-mannosidase, β-D-xylosidase, endomannanase and mutant β-D-mannosidase enzymes) are sensitive for pH-changes and became precipitated during the synthesis

steps using the Kim's method [3]. Although using a one pot process to stabilize enzymes as single enzymes nanoparticles (Yan's method [4]), the stabilization was successful. Size distribution of EM enzyme shows that after each step of stabilization process the size of the enzyme nanoparticles change from 6 nm to 9 nm and 11–12 nm.

We can compare the specific activities of the obtained nanoparticles, the results show that in the case of TMS layer the specific activity is 20% of the native enzyme at the start of the incubation. In the case of SENs with AA layer its activity is 47% of the native CK enzyme at the start of incubation.

The stability of SEN β-xylosidase is higher than that of the non-modified enzyme at 4 °C (with TMS) and 80 °C (with AA). While the non-modified enzyme lost its activity in 15 min at 80 °C without stirring, the SEN–XI remains 40% of its activity after 6 hour incubation. Both types of layers are able to increase the β-xylosidase heat stability even at 80 °C. The heat stability of β-mannosidase (with AA) and cellulase enzyme complex (CK with TMS and AA) was investigated at 80 °C without stirring. Whereas the non-modified enzymes lost their activity after a 0.5-1 hour time period incubation at 80 °C, the stabilized enzyme complexes (MN with AA, CK with TMS and AA) still possess their activities after a 6 hour time period incubation. β-mannosidase enzyme (stabilized with AA) has kept the 40% of its initial activity. Cellulase enzyme complex (CK) with AA layer showed a slightly better stability at 80 °C during 12 hour incubation period, since its starting actvity has reduced only to 50%.

ABBREVATIONS

AA	acrylamide-bisacrylamide
AOT	sodium bis(2-ethylhexyl) sulfosuccinate (aerosol OT)
CK	Celluclast BG cellulase enzyme complex (Novozymes)
EM	endomannanase
MM	mutant mannosidase
SEN	single enzyme nanoparticles
MN	β-mannosidase
NCK	cellulase enzyme nanobiocomposites from CK (method Kim et al.)
NCK_A	cellulase enzyme complex nanobiocomposites from CK (method Yan et al.)
TEM	transmission electron microscope
TMS	methacryloxypropyltrimethoxysilane, or trimethoxysilyl functional group
XI	β-xylosidase

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