2. STABILITY OF CHITIN DEACETYLASE

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1. Introduction

Chitosan is produced on an industrial scale by chemical deacetylation of chitin, but polymer with high acetylation degree is usually obtained as a result of this process. Chitosan with a lower acetylation degree can be produced either by repeating the deacetylation process several times or by conducting the process in stronger conditions. Unfortunately, this leads to parallel hydrolysis of the chitin/chitosan chains [1]. This disadvantage has lead to the investigation of new methods of chitosan production for several years: chitosan separation from fungal cell walls (Zygomycetes) [2 - 4] or enzymatic deacetylation of chitin [5 - 7]. The enzymatic process can be also carried out to lower acetylation degree of chitosan without changing its molecular weight [8]. This process is difficult to carry out chemically.

Enzymatic deacetylation of chitosan is carried out with an enzyme - chitin deacetylase. This enzyme is present in the cells of Mucoraceae fungi (Mucor, Absidia strains) where it takes part in the formation of chitosan in their cell walls [9]. Chitin deacetylase hydrolyses the bond between acetyl and amine groups in the mers of GlcNAc:

$$\text{- GlcNAc} \xrightarrow{\text{Chitin deacetylase}} \text{- GlcN} + \text{AcOH}$$

Chitin deacetylase has been used to date as a native enzyme. Our earlier investigations focused on immobilization of the enzyme allowed us to select a proper carrier and method for immobilizing chitin deacetylase. The best results were obtained for DEAE-Granocel, a carrier with a cellulose matrix [10].

The stability of the activity is one of the most important parameter of enzymes used on an industrial scale. The aim of the presented work was to compare the activity of

the native enzyme separated from Absidia orchidis fungus and immobilized on DEAE-Granocel during 30-days storage at a lower temperature (4-10 °C), in frozen form (-20 °C) and liophilized.

2. Materials and methods

2.1. Chitin deacetylase

Absidia orchidis vel coerulea NCAIM F 00642, kindly donated by the National Collection of Agricultural and Industrial Microorganisms (Hungary), was grown in a batch culture. Biomass was separated from the fermentation medium (centrifugation, 6000 rpm) and frozen. Next, the biomass was slowly melted and homogenized. A crude cell extract was separated from the solid residues by centrifugation (6000rpm, 40 min, 4 °C) and salted out with ammonium sulfate. The solution was stored overnight at 4 °C and protein agglomerates were separated by filtration (0.45 μm, 4 - 10 °C). The clear solution was dialysed and utrafiltrated for desalting and concentration. This solution was used as a native enzyme as well as for the immobilization of chitin deacetylase on DEAE-Granocel [10].

2.2. Chemicals

Chitosan with a medium molecular weight (viscosity of 1% solution of chitosan in 1% acetic acid solution at 25 °C μ = 85 mPa×s, according to data from the producer), with a 27% degree of acetylation (evaluated on the basis of IR spectrum), kindly donated by Gilett-Mahtani-Chitosan (France-India), was used in all experiments.

All other chemicals were analytical grade and purchased by POCH (Poland).

2.3. Activity measurement

Activity of the native chitin deacetylase: Chitosan was dissolved in 1L HCl solution pH 4.0; a final concentration of the solution was of 5 g/L. 100 mL of chitosan solution and 5 mL of free enzyme (in HCl, pH 4,0) were preheated separately for 5 min at 50 °C. The reaction was initiated by adding the enzyme into polymer solution and was carried out at 50 °C in a stirred (250 rpm) thermostated, closed batch reactor. At proper time intervals, the reaction mixture was sampled (2 mL) and the reaction was terminated immediately by the addition of 0.15 mL 1 M NaOH. Then, after separation of precipitated chitosan (centrifugation, 3000 rpm, 30 min), the resulting acetic acid concentration was measured.

Activity of immobilized preparation: 50 mL of the chitosan solution (5 g/L in HCl pH 4.0) and 2 - 3 mL of freely settled carrier (in HCl, pH 4.0) with a total volume of 5 mL were preheated separately for 5 min at 45 °C. A reaction was initiated by adding the enzyme into a polymer solution and carried out at 45 °C in a stirred (250 rpm) thermostated, closed batch reactor. Samples of 2 mL were withdrawn at proper time intervals. The reaction was stopped immediately

with the addition of 0.15 mL 1 M NaOH. Then, after separation of the precipitated chitosan, the resulting acetic acid concentration was measured.

The reaction rate was measured according to the initial rate method.

One activity unit (U) was defined as the amount of the enzyme (μg for a native enzyme or mL of a settled carrier) that increases of acetic acid concentration of 0.1 μmol/mL per 1 minute.

Analytical methods
Protein concentration was measured according to the colorimetric Bradford method using a ready-made kit produced by BioRad (USA, cat no. 500-0006). Acetic acid concentration The clear solution was analyzed using the HPLC method: isocratic system (Waters 625 LC System) with HyperREZ XP Organic acid column (60 °C) with HyperREZ XO Carbohydrate H^+ Guard Column, 0,0025M H_2SO_4 as eluent (0.5 mL/min), and refractometer detector (Waters 410 Differential Refractometer). The quantification limit was evaluated at 5 nmol/mL with a standard deviation of 8% of the mean value.

The method was validated for acetic acid determination in chitosan-HCl (pH 4.0) solutions.

3. Results

Chitin deacetylase is an enzyme that is deactivated by the organic acids: acetic acid, citric acid, formic acid and others. Due to this phenomenon, none of the buffers leaning on these acids can be used. In our earlier investigations, we found that the HCl solution is less harmful to the enzyme in comparison with other buffers tested and HCl solution of pH 4.0 (optimal pH for chitin deacetylase) was used in all experiments.

The aim of the presented work was to check the influence of different methods of enzyme storage on the activity of native chitin deacetylase and enzyme immobilized on DEAE-Granocel. Changes in the activity of native chitin deacetylase during long-term storage at a lower temperature were also investigated.

Influence of the method of storage
The native chitin deacetylase and enzyme immobilized on DEAE-Granocel were stored at a low temperature (4 - 10 °C) as well as frozen (-20 °C) for 30 days or lyophilized (-24 °C) and then stored at 4 - 10 °C. The comparison of activities is presented in Table 1.

It can be clearly observed that the immobilized enzyme became completely inactivated during storage in tested conditions. Contrary to the immobilized form, the native enzyme retained its activity after storage with all tested methods, but its activity decreased in comparison with the initial value. The poorest results were observed for storage at

the lower temperature (4 - 10 °C), although this method is the most common in short-term storage; after 30-day storage, the activity of chitin deacetylase was only of 6.5% of the initial value. The best results were obtained with the liophilized preparation; after 30 days the enzyme retained 33% of initial activity. Storage in frozen form (at -20 °C) also failed to protect chitin deacetylase from inactivation.

Table 1. *Comparison of activity of native and immobilized enzymes.*

	Specific activity	
	native enzyme	enzyme immobilized on DEAE-Granocel
After isolation/ immobilization	12.3 U	3.1 U
Stored at 4 - 10 °C, 30 days	0.8 U	0.0 U
Stored at –20 °C, 30 days	1.8 U	0.0 U
Liophilized at –24 °C, Stored at 4 - 10 °C, 30 days	4.1 U	0.1 U

Influence of storage at lower temperatures

A native chitin deacetylase in HCl solution (pH 4.0) was placed in a fridge at a temperature of 4 - 10 °C. Every few days, the enzyme was sampled and its activity was determined, Figure 1.

The activity of native chitin deacetylase decreased consecutively: after 5 days it was only 65% of initial activity and this figure dropped to 50% after 16 days. Further storage caused nearly complete inactivation of chitin deacetylase: after 30 days its activity was 6.5% of the initial value and stayed at this level for the next 20 days.

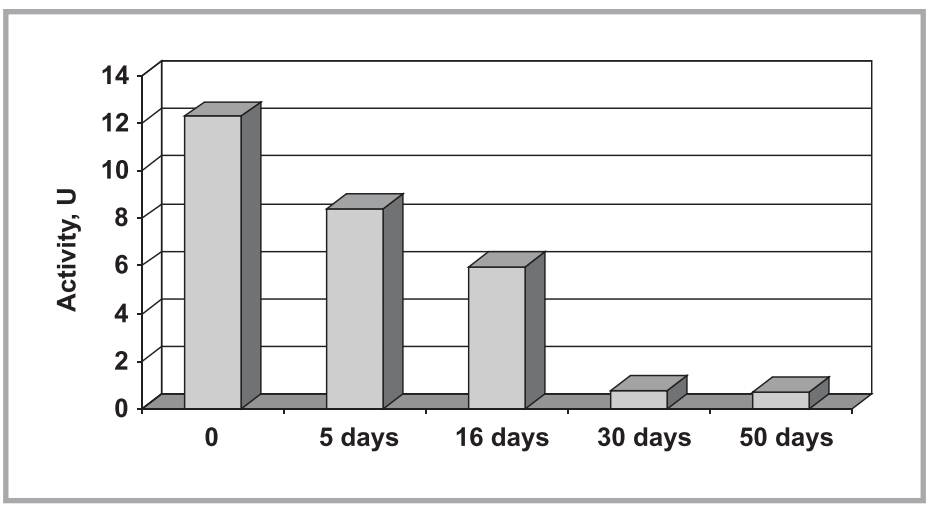

Figure 1. *Changes of activity of chitin deacetylase during long-term storage at a temperature of 4 - 10 °C.*

4. Conclusions

Possible industrial application of chitin deacetylase for chitin or chitosan enzymatic deacetylation requires the development of a method for long-term storage of the enzyme that will not influence its activity. The presented paper was the first step in evaluating the proper method. In our experiments, three different methods of chitin deacetylase storage were tested: storage at a lower temperature, freezing and liophilization. This last determined method was provided the best results, although the activity of chitin deacetylase after 30 days in storage was only 33% of the initial value. Other tested methods obtained poorer results.

It was also shown that immobilization of chitin deacetylase on DEAE-Granocel did not give the expected results. Preparation become completely inactivated in all tested methods, which suggests that immobilization did not protect chitin deacetylase against inactivation.

Further experiments will be carried out to work out a proper method of storage that will retain the activity of chitin deacetylase during long-term storage.

5. References

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