# REACTOR WITH A PRODUCT SEPARATION FOR ENZYMATIC DEACETYLATION OF CHITOSAN

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**Abstract**

The aim of the presented work was to test a reactor with a product separation, membrane reactor for enzymatic deacetylation of chitosan and to compare it with data obtained in a batch process. In our investigations we used chitin deacetylase separated from *Absidia orchidis* vel *coerulea*.

It was shown that the batch process has limited application due to the inhibition of chitin deacetylase by the acetic acid formed during the deacetylation process. In the membrane reactor this phenomena was not observed. It was presented that chitin deacetylase is sensitive to shear stress caused by the pomp used in the membrane reactor set up.

**Introduction**

Chitin deacetylase is an enzyme that hydrolyses the linkage between amine and acetyl groups in N-acetylglucosamine mers. This process can be used in enzymatic deacetylation of chitin or chitosan. Due to the firm crystal structure of chitin, the enzymatic deacetylation of this polymer is still an open problem. Chitosan can be much easier deacetylated enzymaticly because of the easier access of GlcNAc mers for the enzyme, especially in the systems where chitosan is dissolved in a proper solvent.

Chitosan with control acetylation degree can be produced in a two-step process: in the first step chitin is deacetylated chemically to obtain a chitosan with a high degree of acetylation. In the next step, the polymer is dissolved in a proper buffer and then deacetylated enzymaticly with chitin deacetylase.

Chitin deacetylase is an enzyme that can transfer N-acetylglucosamine mers into glucosamine mers:

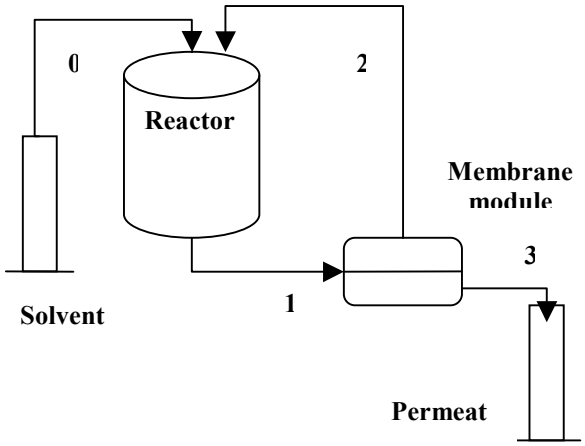
$$\text{- GlcNAc} \xrightarrow{\text{chitin deacetylase}} \text{- GlcN} + \text{AcOH}$$

Unfortunately, the enzyme is inhibited by acetic acid [**1**, **2**] that is released during enzymatic deacetylation. Due to this phenomenon acetic acid must be separated from the reaction mixture continuously. The "membrane reactor" is one of the possibilities. The name "membrane reactor"

describes a reactor connected with a membrane module where products of the reaction can be removed (Schem 1.) and with continuous dosing of the substrate or solvent to stabile the reaction volume.

The aim of the presented work was to test the reactor with product separation in the process of enzymatic deacetylation of chitosan. The membrane reactor was used in our investigations. The polymer was dissolved in HCl solution and was placed in the reactor with chitin deacetylase. The solution was circulated (the pump was placed between reactor and membrane module, not shown at the Sch.1.) through a membrane module and acetic acid formed during the enzymatic reaction was separated through. The pores of the membrane were chosen to be large enough to let the acetic acid pass through and small enough to prevent from leakage of the chitin deacetylase from the reaction mixture.

Chitin deacetylase separated from *Absidia orchidis* vel *coerulea* NCAIM F00642 was used. The experiments were carried out in the conditions optimal for chitin deacetylase: temperature $50^0$C, pH – 4,00 and HCl as a solvent for chitosan. The results were compared with the date obtained for the batch reactor.




**Scheme 1.** The idea of a membrane reactor

## Materials and Methods

*Chitin Deacetylase*

Chitin deacetylase was separated from *Absidia orchidis* vel *coerulea* NCAIM F 00642. The fungi were cultivated in a 7,0-L batch culture ($26^o$C, pH 5,5, YPG nutrient medium [**3**] and separated from the nutrient medium by centrifugation (6000 rpm). Next the biomass was frozen. After thawing, biomass was homogenised and the crude cell extract was separated (centrifugation, 6000 rpm) and salted out with ammonium sulphate (80% saturation) overnight at $4^0$-$6^0$C. The solution was dialysed with HCl (pH 4.0) to remove ammonium sulphate (a membrane module with cut-off 10 kDa) and next concentrated by utrafitrated. The enzyme solution in HCl pH 4.0 was used in experiments.

*Chemicals*

Chitosan with a medium molecular weight (viscosity of 1% solution of chitosan in 1% acetic acid solution at $25^0$C $\mu$ = 85 Pa × s, according to data from the producer), with a degree of acetylation of 39.8% (evaluated on the basis of IR spectrum and with correlation of Domszy and Roberts [**4**]), kindly donated by Gilett-Mahtani Chitosan (France-India), was used in all experiments. The solution of the concentration of 1.0 g/L was used in all experiments.

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

*Batch reactor*

1000 mL of the chitosan solution and 240 µg of chitin deacetylase were preheated separately for 5 min in 50 °C. The reaction was initiated by adding the enzyme into polymer solution and was carried out at an optimal temperature (50°C) in a stirred (200 rpm), thermostated reactor of the volume of 1300 mL. The cover of the reactor was additionally heated to prevent condensation of the vapors on it.

In the proper time intervals the reaction mixture was sampled (2 mL) and the reaction was stopped immediately by adding 0.10 mL 1.0 M NaOH. The precipitated chitosan was separated by centrifugation and then the released acetic acid concentration in a clear solution was measured.

*Membrane reactor*

The membrane reactor was prepared in the same way as the batch reactor, but it was additionally connected with a Vivaflow membrane module (Sartorius, Germany) with a cut-off 10 kDa and area

of 50 $cm^2$. The circulation (Masterflex pomp) of the solution through the membrane module was started 5 min after initiation of the reaction. The loss of the solvent due to permeation through the membrane was replaced by HCl solution (pH 4.0) at a rate equal to that of permeation.
In the proper time intervals the reaction mixture was sampled (2 mL) and the reaction was stopped immediately by adding 0.10 mL 1.0 M NaOH. The precipitated chitosan was separated by centrifugation and then the released acetic acid concentration in a clear solution was measured.

*Analytical methods*

Protein concentration was determined according to the Bradford method using a ready-made reagent of Biorad (USA, cat. No. 500-0006) and bovine serum albumine as a standard.
Acetic acid concentration in the clear solution was analyzed using the HPLC method: isocratic system (Varian ProStar 210) with HyperREZ XP Organic acid column (60°C) and HyperREZ XO Carbohydrate $H^+$ Guard Column, 0,0025M $H_2SO_4$ as eluent (0.5 mL/min), and refractometer detector (Varian ProStar 350). 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.

**Results and Discution**

In all the experiments chitosan solution in HCl (pH 4.0) with the concentration of 1,0 g/L and 240µg of chitin deacetylase was used. Experiments were performed in conditions optimal for the enzyme: $50^0$C and pH 4.0.

*Enzymatic deacetylation in a batch reactor*

The changes of the acetic acid concentration in the reaction mixture are presented in Fig. 1.

It can be easily observed that the increase in acetic acid concentration was the fastest during the first hours of the experiments. After 4 hours it reached its maximal value. Continuation of the process didn't influence the acetic acid concentration significantly.

The presented data can be explained by the inhibition of chitin deacetylase by the product of the reaction. Enzyme became deactivated by acetic acid formed during the reaction.
The total amount of acetic acid released in the 6-hour experiment was 298.5 µmol and corresponded with the 6% change in the acetylation degree.

*Enzymatic deacetylation in a membrane reactor*

The membrane reactor can be suitable for enzymatic deacetylation of chitosan because it provides an opportunity to remove acetic acid from the reaction mixture during the process and to prevent inactivation of the enzyme by the product of the reaction. Due to the application of the pump for circulation of the reaction mixture through the

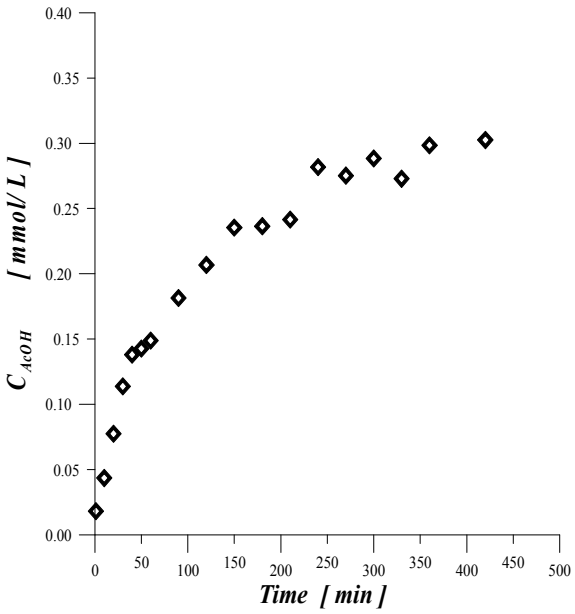



**Figure 1.** Changes in the concentration of acetic acid in a batch reactor

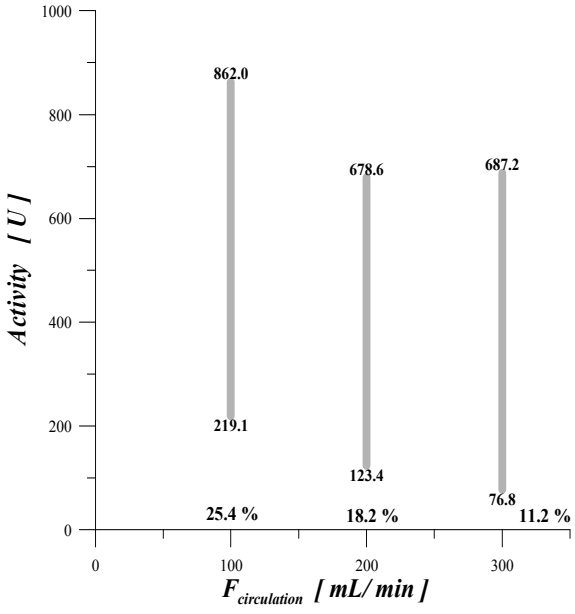



**Figure 2.** Changes in the activity of chitin deacetylase

membrane module, influence of the shear stress caused by the pump on the enzyme was tested first. The cell crude extract separated from the fungi were placed in a beaker and the liquid was circulated with a constant speed (100 mL/min, 200 mL/min, 300 mL/min) for 24 hours. The initial activity of the chitin deacetylase was compared with the activity of the same solution after 24 hours. Results are presented in Fig 2. (higher values correspond to initial activity, lower to final activity; percentage of the initial activity is also shown).
The deactivation of the chitin deacetylase due to sheer stress of the pump can be easily observed. When increasing the circulation rate the decrease in activity is more significant. Increasing the circulation rate to 300 mL/min caused decrease in the activity of the chitin deacetylase to 11% after 24 hours, while at a circulation rate equal to 100 mL/min it remained 25%. This means that there is a strong limitation in the circulation rate.
The circulation rate 100 mL/min was chosen for all presented experiments.

In the next step of experiments, the influence of chitosan on the distribution coefficient and permeation rate were investigated. A solution without (HCl + AcOH) or with chitosan (HCl+Chitosan 5g/L + AcOH) was circulated in the set up and the changes in the acetic acid concentration were measured.
The distribution coefficient (*R*) was defined as the ratio of the concentration of acetic acid in permeat (stream 3) to the concentration of acetic acid in the stream leaving the membrane module (stream 2, see Scheme 1.)
On the basis of the mass balance of the set up (eq. 1):

$$-V\frac{dC_{AcOH,1}}{dt} = F_0 C_{AcOH,0} - F_3 C_{AcOH,3} \quad (\mathbf{1})$$

where: $V$ – volume of the reaction mixture, mL; $F$ – flow rate of a proper stream, mL/ min; $C_{AcOH}$ – concentration of acetic acid, μmol/L; subscripts $_{0,\,1,\,3}$ – corresponds to the streams as in the Sch. 1.
with the approximation that the concentration of acetic acid in stream 2 is nearly the same as in stream 1 (hypothesis was verified analytically)

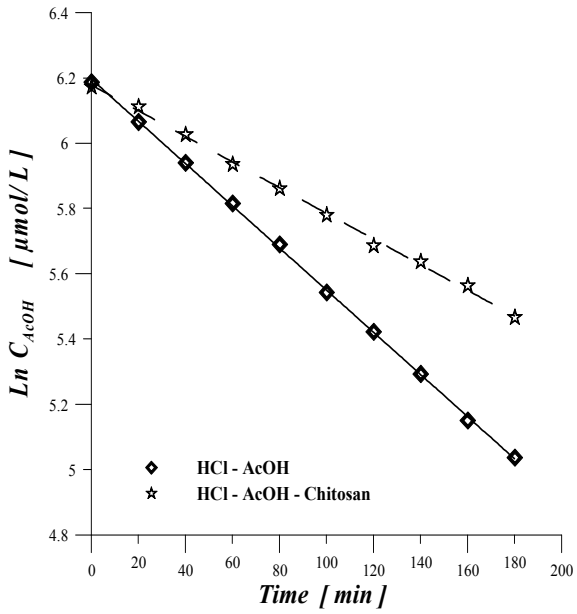



**Figure 3.** Estimation of distribution coefficients

$$R = \frac{C_{AcOH,3}}{C_{AcOH,2}} \approx \frac{C_{AcOH,3}}{C_{AcOH,1}} \quad (\mathbf{2})$$

and for $C_{AcOH,\,0} = 0$ , with the bordery conditions: $t=0$ and $C_{AcOH,1} = C^0_{AcOH,1}$
we will get the analytical solution:

$$Ln(C_{AcOH,1}) = Ln(C^0{}_{AcOH,1}) - \frac{F_3 R}{V} t$$

The distribution coefficient (*R*) can be estimated on the basis of the plot $Ln(_{AcOH,1})$ *vs t* where the $tg\alpha = -F_3R/V$, Fig.3. Their values are presented in Tab. 1.:

**Table 1.** The distribution coefficients estimated for the membrane reactor

| | $F_1$ | $F_3$ | R |
|---|---|---|---|
| | mL/min | mL/min | |
| HCl + AcOH | 100 | 5.8 | 1.12 |
| HCl + Chitosan + AcOH* | 100 | 3.8 | 1.05 |

volume of the reaction mixture V = 1000 mL
* - 5 g/L of chitosan in HCl solution (pH 4.0)

As it can be easily observed, the addition of the chitosan did not influence the value of the distribution coefficient but significantly decreased the stream of permeat $F_3$; its value decrease nearly one and a half times.

The results of the enzymatic deacetylation of chitosan by chitin deacetylase in a membrane reactor are presented in Fig. 4

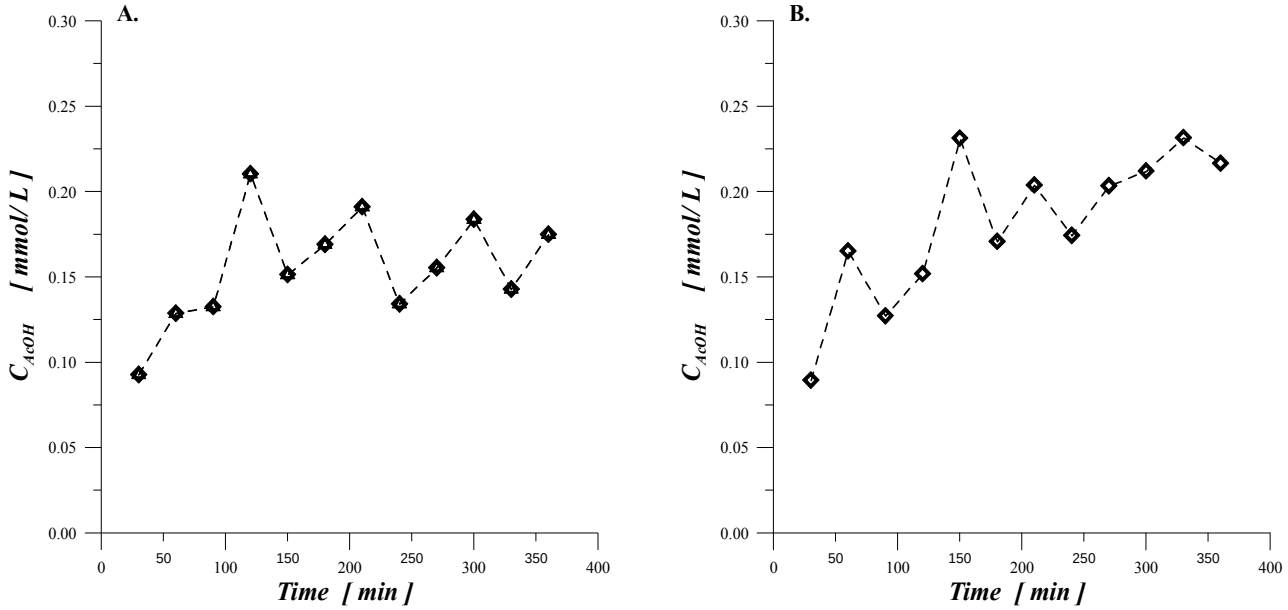



**Figure 4.** Changes in acetic acid concentration within the reactor (**A.**) and in the permeate (**B.**)

The concentration of acetic acid in the reactor increased slowly during the first 2-3 hours and after that oscillations were observed. The final concentration of AcOH was 175 μmol/L. At the same time, the concentration of acetic acid in the permeat increased continuously and after 6 hours it reached 217 μmol/L (permeation rate 3.5 mL/min). The total amount of acetic acid released during the 6-hour experiment was 287.5 μmol and corresponded to the expected change in the acetylation degree of the chitosan equal to 5.9%. The final result of the 6-hours experiment was similar to the process carried out in a batch reactor, but contrary to the batch process we didn't observe inactivation of the chitin deacetylase by acetic acid formed during the reaction. The inhibitor was successfully removed from the reaction mixture and the reaction was not stopped by its activity.

**Conclusion**

The process of enzymatic deacetylation of chitosan can be used to modify the acetylation degree of the polymer without changing its molecular weight. Because chitin deacetylase is inhibited by acetic acid, the product of the reaction, a decrease of AD in the batch reactor is limited.

Therefore it is necessary to remove acetic acid from the reaction mixture. The membrane reactor seemed to be one possible solution.
In the presented paper we compared the process carried out in a batch reactor with that in a membrane reactor. In experiments lasting 6 hours we obtained similar results, but contrary to the batch process we didn't observed the inhibition of chitin deacetylase by acetic acid released in the reaction. It was also shown that chitin deacetylase is sensitive to shear stress caused by the pump used to circulate the reaction mixture through the membrane module. This phenomenon must be taken into account in further investigations.

As a final conclusion we can state out that the membrane reactor offer better condition for enzymatic deacetylation of chitosan than the batch reactor.

**References**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