ENZYMATIC DEACETYLATION OF CHITOSAN/CHITIN FROM A THERMODYNAMIC POINT OF VIEW

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Abstract

The paper presents thermodynamic studies on enzymatic chitosan deacetylation based on the action of intracellular chitin deacetylase (EC 3.5.1.41) from Absidia orchidis vel coerulea. Based on those studies, the entropy and enthalpy of formation of the enzyme-substrate complex (ES; $\Delta H° = 81.386$ kJ/mol, $\Delta S° = 220.73$ J/(mol·K)) as well as product formation (P+E; $\Delta H^{\#} = 75.15$ kJ/mol, $\Delta S^{\#} = 78.13$ J/(mol·K)) were determined. Based on their values, the Gibbs free energy for each step was calculated. It was shown that neither the formation of the enzyme-substrate complex (ES) nor the formation of the product (P) is spontaneous in the investigated range of temperatures (35–50 °C). Possible reasons for the lack of spontaneity and for the comparison with the chemical process were discussed in the paper.

Keywords: *chitin deacetylase, chitosan, chitin, enthalpy, entropy, Gibbs free energy*

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1. Introduction

Although the enzymatic deacetylation of chitin/chitosan has been investigated since the early 90s of the 20th century, thermodynamic data of the process are still missing. The values of enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG) are not available in either literature or thermodynamic databases. Knowing them, it is possible to assess whether the process is spontaneous. These data, especially enthalpy and entropy, are also important when one wants to change the conditions of the process, as several data can be recalculated without the necessity of conducting experiments. Additionally, these thermodynamic functions are functions of the state, so their values do not depend on the mode of action but only on the initial state of the substrate and the final state of the products. It means that they can be used not only when the investigated enzyme is used, but also with any enzyme used for the enzymatic deacetylation of chitin/chitosan.

Chitin deacetylase, CDA (EC 3.5.1.41), is an enzyme that is known for its ability to convert *N*-acetylglucosamine (GlcNHAc) units in the chitosan or chitin chain into the glucosamine ($GlcNH_2$) units (Scheme 1):

$$-\mathrm{GlcNHAc} + \mathrm{H_2O} \xrightarrow{\text{chitin deacetylase}} -\mathrm{GlcNH_2} + \mathrm{AcOH}$$

Scheme 1. Enzymatic deacetylation of the *N*-acetylglucosamine unit.

The process is based on the hydrolysis of the acetamido bond and is called deacetylation. The degree of deacetylation (DA, content of *N*-acetylglucosamine units in a chitin or chitosan chain) influences the physico-chemical and biological properties of the biopolymer. With a decrease in DA, the properties of chitosan become more favourable: its solubility in acidic water-based solutions increases, which is particularly important for applications in agriculture or the food industry [1–3]. Moreover, chitosan with a low acetylation degree shows higher bioactivity and stronger antimicrobial properties [4–6].

Chitosan with a low acetylation degree is produced by the chitin or chitosan deacetylation process, which is carried out using concentrated NaOH solutions (40–50%) at elevated temperatures. The hydrolysis of the polymer chain is also observed. Unlike the chemical method, the enzymatic method is carried out under mild conditions (40–55 °C) and yields a polymer with an unchanged molecular weight, as the enzyme chitin deacetylase does not cause chitosan chain depolymerisation [7]. This enzyme is produced by several microorganisms (bacteria and fungi), but regardless of the source of the enzyme, the process leads to chitosan with a lower degree of acetylation. The properties of chitin deacetylase (optimal pH, temperature, and kinetics) are well documented in the literature.

The reaction of enzymatic deacetylation of chitosan can be described with the Michaelis-Menten equation (Equation 1):

$$\upsilon = \frac{k_{cat} \cdot C_e \cdot C_S}{K_M + C_S} \tag{1}$$

where:

υ – reaction rate [mol/(L·min)];
k_{cat} – reaction rate constant [1/min];
C_e – enzyme concentration [mol/L];
C_S – substrate concentration [mol/L];
K_M – Michaelis constant [mol/L].

Kinetic parameters of this equation, υ_{max} (= $k_{cat} \cdot C_e$), k_{cat}, and K_M, have been published for chitin deacetylases, but data were presented usually for optimal pH and only one (usually optimal) temperature. For example, Tokuyasu *et al.* [8] using CDA from *Colletotrichum lindemuthianum* ATCC 56676 with $(GlcNHAc)_5$ as a substrate evaluated K_M = 69.9 μM and k_{cat} = 53.1 1/s at 45 °C. For CDA from *Aspergillus nidulans* CECT 2544, the kinetics parameters K_M = 2.50 mM, υ_{max} = 33.3 nmol/min for $(GlcNHAc)_5$ as substrate were evaluated at 37 °C [9]. Jaworska [10] investigated intracellular CDA from *Absidia coerulea* with chitosan of DA = 39.8% as a substrate. Kinetic parameters were evaluated as K_M = 627 mg/L, k_{cat} = 0.667 1/min at 50 °C. To the best of our knowledge, the influence of temperature on those parameters has not yet been presented in the literature. Knowing the influence of temperature on K_M and k_{cat}, the thermodynamic functions could be evaluated.

The hypothesis put forward in this work is that a thermodynamic study would help elucidate the action of chitin deacetylase and the difference between enzymatic and chemical deacetylation of chitin/chitosan. In the present work, the enthalpy (ΔH) and entropy (ΔS) thermodynamic functions of the enzymatic deacetylation of *N*-acetylglucosamine units in the chitin/chitosan chain were evaluated. These functions were not related to chitosan/chitin concentration but to GlcNHAc units' concentration [10], as these units are the substrate for the enzyme. The intracellular chitin deacetylase from *Absidia orchidis* vel *coerulea* was used.

2. Materials and Methods

2.1. Chitin Deacetylase

The enzyme separated from the biomass of *Absidia orchidis* vel *coerulea* NCAIM F 00642 was used in experiments (the enzyme is not commercially available). The fungi were cultivated in a 5.0-L batch culture (26 °C, pH 5.5, YPG nutrient medium [11]) and separated from the nutrient medium by centrifugation (6000 rpm (4400g), 20 min). Next, the biomass was frozen, then slowly thawed (+5 °C) and homogenised. The crude cell extract was separated by filtration (0.45 μm) and salted out with 80% ammonium sulphate (2 h, room temperature). The solution was dialysed with HCl (pH 4.0) to remove ammonium sulphate (using a 10 kDa cut-off PES membrane module, Sartorius) and then concentrated by ultrafiltration (the same membrane module). The enzyme solution of pH adjusted with HCl to 4.0 (optimal pH) was used in all experiments. All separation operations were performed at room temperature unless otherwise stated.

The concentration of the proteins was equal to C_e = 27.03 μg/mL and was assumed to be the concentration of the enzyme (C_e = 0.386 μmol/L, molecular weight of the enzyme 70 kDa).

2.2. Chitosan

Chitosan (CAS no. 9012-76-4) from shrimps (BioLog Heppe, Germany) with a degree of acetylation (AD) of 39.2% and medium molecular weight (500 kDa), according to the information of the producer, was used in all experiments. Chitosan (4.0 g) was mixed with 800 mL of pure water, and HCl solution (1%) was added dropwise under pH-controlled conditions (4.0 ± 0.1). The solution was filtered on paper filters of 1 μm cut-off to remove insoluble residues. The solution was enhanced to 1000 mL with HCl (pH 4.0). The concentration of the prepared chitosan solution was confirmed by the gravimetric method.

Lower concentrations of chitosan were prepared by mixing the concentrated solution with HCl (pH 4.0) in volumetric flasks. For kinetic studies purpose, chitosan concentration was recalculated from mass concentrations to molar concentrations of GlcNHAc. The calculations were based on the DA of chitosan and the molecular mass of GlcNHAc units (219.21 g/mol) and $GlcNH_2$ units (177.17 g/mol).

2.3. Reaction Rate

The reaction rate of the enzymatic deacetylation of chitosan was determined using the initial rate method. 50 mL of chitosan solution of known concentration was added to a 150 mL reactor and incubated at the temperature of the reaction (35–50°C) for 10 minutes, with continuous stirring (200 rpm). 2 mL of chitin deacetylase solution (preheated at the same temperature for 2 min) was added to initiate the reaction. At appropriate time intervals (after 15, 30, 45, 60, 120, 180, 300, 420, 600, and 900 s), 2 mL samples were collected and immediately mixed with 0.1 mL of 1 M NaOH to stop the reaction. Samples were then centrifuged (5000 rpm (approx. 1600g), 10 min) to separate the precipitated chitosan. The supernatant was collected and filtered (cellulose nitrate syringe filters with a cut-off of 0.45 μm). The concentration of acetic acid was determined for each sample.

The reaction rate was calculated as the arithmetic mean of the results obtained from duplicate reactions and was deemed acceptable when the differences between the results did not exceed 10%.

2.4. Analytical Methods

The concentration of acetic acid was analysed using the HPLC method, as described by Jaworska [10]. The HPLC system consisted of a HyperREZ XP Organic Acid Column and a HyperREZ XO Carbohydrate H^+ Guard Column (at 60 °C). The isocratic system, Varian ProStar 210, was used with a refractometric detector (Varian ProStar 350) and deionised water (0.5 mL/min) as the eluent. The quantification limit was evaluated at 5 nmol/mL, with a standard deviation of 8% of the mean. The method was validated for the determination of acetic acid in chitosan-HCl solutions.

Protein concentration was evaluated using Bradford's microassay method with Coomassie Brilliant Blue (Bio-Rad, USA). The calibration curve was prepared using bovine serum albumin as a standard.

3. Results

The enzymatic deacetylation of *N*-acetylglucosamine units in chitin and chitosan can be described with the Michaelis-Menten equation [8–10]. The parameters in this equation (K_M and k_{cat}) are correlated with the thermodynamic Gibbs function (ΔG) and then, according to the ΔG definition, with enthalpy and entropy. Thus, by investigating the influence of temperature on kinetic parameters, values of ΔH and ΔS can be evaluated. Experiments were carried out over the temperature range of 35 to 50 °C, where the thermal deactivation of the enzyme does not influence the kinetic results when the initial rate method is applied.

According to the Michaelis-Menten model, the scheme of a simple enzymatic reaction proceeds as shown in Scheme 2.

$$E + S \overset{K_M}{\leftrightarrow} ES \overset{k_{cat}}{\rightarrow} P + E$$

Scheme 2. Kinetic scheme of enzymatic reactions.

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In the first step, the reversible reaction between the substrate (S) and the enzyme (E) leads to the formation of the enzyme-substrate (ES) complex. This first step can be considered as an equilibrium state and is described by the Michaelis constant (K_M), which can be treated as a reaction equilibrium constant according to Michaelis-Menten theory [12]. The second step, converting the enzyme-substrate complex into the product (P), is irreversible and proceeds with the reaction rate constant, described as k_{cat} [12]. The Gibbs free energy for the transition of the enzyme and substrate (E + S) to the enzyme-substrate complex was defined as $\Delta G°$, and the transition of the enzyme-substrate complex (ES) to product (P) was defined as $\Delta G^{\neq}$. To determine $\Delta G°$, the equation that connects it to the Michaelis constant was used, while to evaluate $\Delta G^{\neq}$, Eyring's transition-state theory was applied [12–15].

3.1. Influence of Temperature on K_M and k_{cat}

In the first step, the evaluation of the influence of temperature on the parameters in the Michaelis-Menten equation was performed. Experiments were carried out at a temperature range of 35–50 °C. Kinetics data are presented in Figure 1. The k_{cat} and K_M were evaluated using original experimental data and the non-linear approximation method (Table 1).

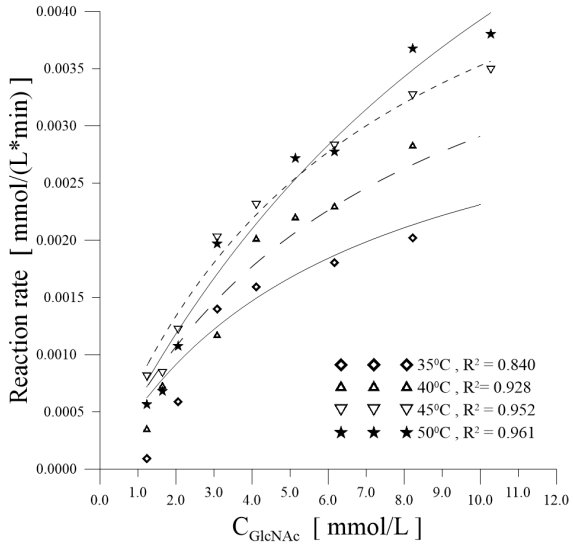


Figure 1. The reaction rate for the enzymatic deacetylation of *N*-acetylglucosamine units at different temperatures.

Table 1. The kinetic parameters of the Michaelis-Menten equation for different temperatures.

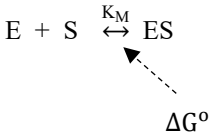
Temperature	K_M [mM]	k_{cat} [1/min]	R^2	k_{cat}/K_M [1/(mM·min)]
35 °C (308.15 K)	6.17	251.7	0.840	40.79
40 °C (313.15 K)	7.51	342.9	0.928	45.66
45 °C (318.15 K)	14.16	620.8	0.952	43.84
50 °C (323.15 K)	25.84	990.1	0.961	38.31

Both parameters of the Michaelis-Menten equation presented in Table 1 increased with rising reaction temperature. For both, the parameters at the highest temperature (50 °C) were approximately 4 times higher than those at the lowest (35 °C). An increase in K_M indicates that the concentration of the substrate needed to achieve a given degree of saturation of the enzyme's active site must be increased significantly with an increase in temperature (at full saturation of the enzyme's active site, the reaction rate approaches maximum and becomes nearly independent of substrate concentration), which can also be observed by looking at the shape of the curves presented in Figure 1. An increase in k_{cat} increases the reaction rate, which can also be observed in Figure 1, although the reaction rate was relatively slow compared to other enzymes.

The calculated turnover number (k_{cat}/K_M) changes slightly with increasing temperature, reaching a maximum at 40 °C. The changes are not significant, as the difference between the highest k_{cat}/K_M value (at 40 °C) and the lowest one (at 50 °C) reaches ca. 19%.

3.2. Thermodynamic Functions of Enzymatic Deacetylation: Formation of the ES Complex

The reaction of an enzyme and substrate in the first step leads to the formation of an enzyme-substrate complex (Scheme 3):



Scheme 3. Reversible formation of the ES complex.

The Gibbs free energy (ΔG°) for the transition of the enzyme and substrate to the enzyme-substrate complex is connected with the Michaelis constant (K_M) treated as an equilibrium constant according to Equations 2 and 3 [15]:

$$K_M = \exp\left(\frac{-\Delta G^\circ}{RT}\right) \tag{2}$$

$$\ln K_M = \frac{-\Delta G^\circ}{R} \cdot \frac{1}{T} \tag{3}$$

where:

R – universal gas constant [8.31446 J/(K·mol)];

T – temperature [K].

Applying the definition of Gibbs free energy ($\Delta G = \Delta H - T\,\Delta S$), the correlation of the Michaelis constant with enthalpy (ΔH) and entropy (ΔS) is obtained according to Equation 4:

$$\ln K_M = \frac{-\Delta H^\circ}{R} \cdot \frac{1}{T} + \frac{\Delta S^\circ}{R} \tag{4}$$

Thus, plotting the $\ln K_M$ vs (1/T), where K_M is expressed in [mol/L], and T is expressed in [K], the values of ΔH° and ΔS° can be evaluated, as presented in Figure 2.

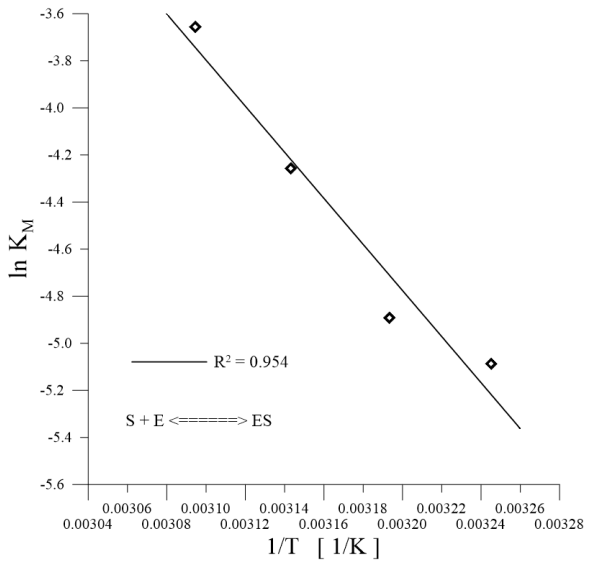
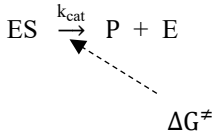


Figure 2. Determination of thermodynamic functions for enzyme-substrate formation.

The obtained data showed a good linearity. The Y-axis intercept equals ΔS°/R, while the slope of the line equals −ΔH°/R. The calculated thermodynamic functions are ΔH° = 81.386 kJ/mol and ΔS° = 220.73 J/(mol·K).

3.3. Thermodynamic Functions of Enzymatic Deacetylation: Formation of the Product

The next step of the reaction is the conversion of the ES complex ES into a product (P) (Scheme 4):



Scheme 4. Irreversible formation of the product P.

Simultaneously, the enzyme (E) is released.

This reaction can be regarded as first-order; thus, according to Eyring's transition state theory [12], the reaction rate constant (k_{cat}) is correlated with $\Delta G^{\neq}$ by Equation 5:

$$k_{cat} = \left(\frac{k_B \cdot T}{h}\right) \exp\left(\frac{-\Delta G^{\neq}}{RT}\right) \quad (5)$$

where:

k_{cat} – reaction rate constant [1/s];
k_B – Boltzmn constant [$1.3807 \cdot 10^{-23}$ J/K];
h – Planck's constant [$6.6261 \cdot 10^{-34}$ J·s];
R – universal gas constant [8.31446 J/(K·mol)];
T – temperature [K].

Applying the definition of Gibbs free energy ($\Delta G = \Delta H - T\,\Delta S$), Equation 5 can be transformed into Equation 6:

$$k_{cat} = \left(\frac{k_B \cdot T}{h}\right) \exp\left(\frac{-\Delta H^{\#}}{RT} + \frac{\Delta S^{\#}}{R}\right) \quad (6)$$

By dividing both sides by T and taking natural logarithms, we obtain:

$$\ln\left(\frac{k_{cat}}{T}\right) = \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^{\#}}{R} - \frac{\Delta H^{\#}}{R} \cdot \frac{1}{T} \quad (7)$$

Thus, plotting the relationship ($\ln(k_{cat}/T)$) vs (1/T) where T is expressed in [K], the values of $\Delta H^{\#}$ and $\Delta S^{\#}$ can be calculated (Figure 3).

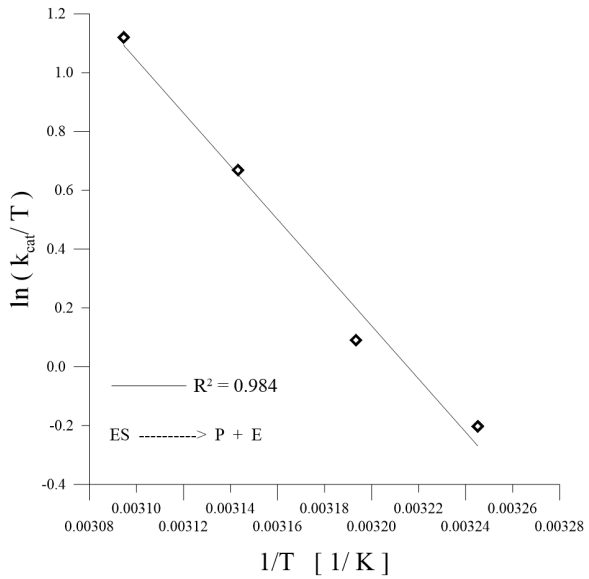


Figure 3. Eyring's plot.

The obtained data also showed good linearity. The intercept of the Y-axis is equal to $(\ln(k_B/h)+(\Delta S^{\#}/R))$, while the slope of the line is equal to $-\Delta H^{\#}/R$. The thermodynamic functions of product formation were evaluated as $\Delta H^{\#} = 75.15$ kJ/mol and $\Delta S^{\#} = 78.13$ J/(mol·K).

4. Discussion and Conclusions

Interest in the enzymatic deacetylation of chitin/chitosan increases every year due to the possible production of chitosan from chitin using the enzymatic method. Such a method would solve the problem of hazardous pollution produced by the chemical method (e.g. strong alkali). Papers on chitin deacetylase mainly focus on the characterisation of the enzyme isolated from different sources. The literature presents data on the structure of the enzyme chain, genes coding the enzyme, and some kinetic data. The only thermodynamic data published so far were focused on the deactivation of chitin deacetylase. Pareek *et al.* [16] evaluated and presented the deactivation energy ($E_{a,d}$ = 114.72 kJ/mol), free energy of deactivation (ΔG_d = 97.86 kJ/mol), deactivation enthalpy (ΔH_d = 112.04 kJ/mol) and entropy (ΔS_d = 43.93 J/(mol·K)) for denaturation

performed at 50 °C. Thus, there was a lack of thermodynamic data on the enzymatic deacetylation of chitin/chitosan by enzymes obtained from any source. This paper is the first to present the influence of temperature on kinetic parameters (K_M and k_{cat}) and thermodynamic parameters (ΔH, ΔS, and ΔG) for the enzymatic deacetylation of GlcNHAc units in chitosan and chitin.

It was interesting to notice that the Michaelis constant (K_M) increased with increasing temperature. K_M value increased approximately fourfold when the temperature was raised from 35 to 50 °C. K_M represents the affinity of the substrate to the active site [12]. Low K_M values indicate high affinity, while high values indicate lower affinity. The data indicate a decrease in the substrate's affinity to the enzyme as the temperature increases. The weak attraction between GlcNHAc and the active site might cause, that the high substrate concentration is needed to achieve full saturation and maximal reaction rates. This could also suggest weaker binding of the substrate to the active site at higher temperatures compared to lower ones. This weakening in bonding could be compensated by an increase in the reaction rate constant k_{cat}. Its value also increases by approximately four times with increasing temperature.

The enthalpy and entropy of both reaction steps were evaluated and were found to be positive. The Gibbs free energy calculated for both steps is presented in Table 2.

Table 2. The Gibbs free energy for the enzymatic deacetylation of chitosan.

Temperature	35 °C (308.15 K)	40 °C (313.15 K)	45 °C (318.15 K)	50 °C (323.15 K)
E + S ↔ ES (step 1)				
ΔG° [kJ/mol]	13.37	12.27	11.16	10.06
ES → P + E (step 2)				
$\Delta G^{\#}$ [kJ/mol]	51.07	50.68	50.29	49.90

At all temperatures and at each elemental step of the deacetylation process, the Gibbs function is positive. Positive ΔG° (10–13 kJ/mol) means that equilibrium favours ES dissociation, but steady state [ES] enables catalysis at [S] ≈ K_M. The ΔG° and $\Delta G^{\#}$ values decrease with increasing temperature, suggesting that there is a temperature above which the functions would become negative, and the reaction would become spontaneous. These temperatures were calculated as 96 °C (369.18 K) for the formation of the ES complex (step 1) and 689 °C (962.15 K) for the reaction of product formation (step 2). These temperatures exceed the temperature at which enzymes become inactivated and are therefore unsuitable for deacetylation.

The values obtained for the thermodynamic function may vary slightly due to impurities in the enzyme used in the experiments. These impurities would influence mainly the values of ΔS° and $\Delta S^{\#}$ (by slightly changing the intercept of the Y-axis), while the values of ΔH° and $\Delta H^{\#}$ would stay unaffected (the unchanged slope of the relationships presented in Figures 2 and 3.

To explain the non-spontaneousity of the enzymatic deacetylation of chitosan, one has to take into consideration possible reasons: conformation of the chitosan chain in the solution, electrostatic interaction between enzyme and chitosan chain, or the role of chitin/chitosan in the living organisms.

It was reported that, depending on the molecular weight of chitosan, its conformation in solution can change from rod-like (for low-molecular-weight biopolymer) to random coil (for biopolymer with higher molecular weight), and that temperature did not influence

the shape of the polymer chains [17, 18]. Commercial chitosan is always a mixture of biopolymers with chains of different lengths, resulting in different structures in the medium. The short chains (rod shape) can be deacetylated faster than biopolymers with longer chains and random coil structure, where GlcNHAc units can be "hidden" inside the coil and are less available for the enzyme.

Another important aspect is the possible electrostatic interactions between the chitosan chains and between the chitosan chain and the enzyme. One must remember that the chitosan chain is built with random blocks of $GlcNH_2$ and GlcNHAc units [19, 20]. In acidic solution (chitosan is soluble below pH = 5.5), the $GlcNH_2$ units are protonated to $GlcNH_3^+$, gaining a positive charge. This charge is responsible for the formation of agglomerates with other chitosan chains, as reported by Amiji [21] and Pedroni *et al.* [22]. This electrostatic interaction is formed between acetylated and deacetylated units. The formation of agglomerates is an additional inconvenience for the enzymatic process, as GlcNHAc units become less available. However, the electrostatic interaction between $GlcNH_3^+$ units and the enzyme should not be excluded. The amino acids present at the outer surface of the 3D structure of the enzyme could also be protonated in reaction conditions, and electrostatic repulsion cannot be excluded. This electrostatic repulsion would be another barrier to overcome.

One must remember the role that chitin and chitosan play in nature - they are major components of the external skeletons of several groups of organisms. They are present in crustacean shells (chitin), insect shells (chitin), and cell walls of yeasts (chitin) or fungi (chitosan, chitin). Thus, these polymers must be chemically resistant so as not to be easily hydrolysed or modified by compounds/enzymes present in the environment. Chitin is composed mainly of GlcNHAc units, which confer the biopolymer's chemical resistance. Due to their role in nature, these units must be not only chemically resistant but also resistant to the action of enzymes produced by pathogens (microorganisms). Otherwise, the shells or microbial cell walls could be easily destroyed.

Currently, the deacetylation of chitin/chitosan is carried out primarily as a chemical process, using concentrated alkali solutions (40–50% NaOH or KOH) at elevated temperatures (above 90 °C). Under these conditions, the highly reactive reactants can readily overcome the energy barrier in the deacetylation process. The conditions of the enzymatic process are mild (temperatures in the range of 40–55 °C), and although the enzyme lowers the energy barrier in the deacetylation reaction, it may be insufficient for the process to be effective. Enzymatic deacetylation is possible, but it would take longer to achieve the same final degree of deacetylation, as the reaction rate would be slower due to, e.g., lower temperatures used. These are the reasons the chemical process remains still more efficient than enzymatic deacetylation.

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6. Author Contributions

Conceptualisation, M.Z., M.M.J., and B.M.S; methodology, M.M.J and B.M.S.; validation, M.Z., B.M.S., and M.M.J.; investigation, M.Z. and B.M.S.; data curation, M.Z. and B.M.S; writing—original draft preparation, M.M.J.; writing—review and editing,

M.Z., B.M.S., and M.M.J.; funding acquisition, M.M.J. All authors have read and agreed to the published version of the manuscript.

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