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Modeling of enzymatic chitosan gelation: tools for monitoring chitosan gelation and urea hydrolysis kinetics

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Abstract

A modeling of urea hydrolysis in chitosan medium and global enzymatic chitosan gelation kinetics was developed to better understand the gelation dynamics. The model was validated using experimental results in batch stirred reactor. Comparison between urea hydrolysis reaction rates in water and chitosan media shows that the chitosan has nearly no influence on the enzymatic kinetics parameters. Modeling results allowed decoupling gelation phenomena and urea hydrolysis reaction and pointing out the limitation of urea hydrolysis reaction by urea concentration depletion in chitosan medium. Monitoring pH and $d(\text{pH})/dt$ was useful for determining (i) the gelation time, (ii) the advanced gelation time, (iii) time of the maximum gelation rate and (iv) time of the maximum urea hydrolysis rate.

Keywords

Chitosan gelation, Gelation pH, Advanced Gelation pH, Gelation rate, Urea hydrolysis rate

Declarations of interest: none

1. Introduction

Chitosan is a bio-based *N-deacetylated polysaccharide* composed of a succession of *D-glucosamine* (GlcN) and *N-acetyl-glucosamine* (GlcNAc) units (Figure 1). A given chitosan is characterized by its degree of deacetylation (**DDA**, expressing the percentage of GlnN units), upper than 60% to differentiate chitosan from chitin, and its weight-average molecular weight (MW_{chit}) between 50,000 and 2000,000 $\text{g}\cdot\text{mol}^{-1}$ [1].

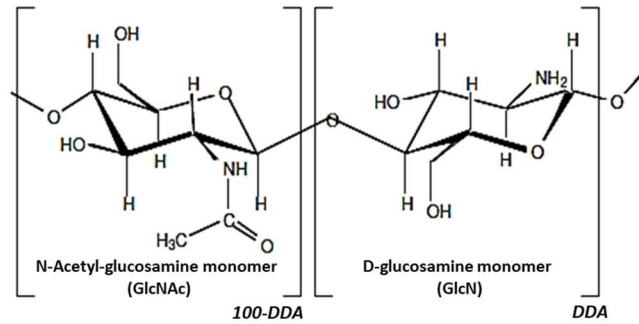


Figure 1: Chitosan *N*-acetyl glucosamine (GlcNAc) and *D*-glucosamine (GlcN) units.

Amino-groups of *D*-glucosamine chitosan units are mostly protonated in acid aqueous medium while they are mostly non-protonated in basic conditions, favoring macromolecular association and resulting in a “physico-chemical” gelation of chitosan in aqueous medium (**Reaction 1**) when pH becomes higher than the pKa of the chitosan amino-groups (pKa = [6.2 - 7.1]) [2 - 7]. In order to prepare chitosan gel, chitosan is usually dissolved in dilute acid aqueous solution such as acetic acid or hydrochloric acid to reach a polymer concentration around 2 to 4% w/v. Then, chitosan gelation occurs due to an external supply of sodium hydroxide or ammonia [8 - 12]. Recently, an original enzymatic chitosan gelation process has been proposed by Chenite *et al.* [13] to obtain homogeneous gelation: the OH⁻ ion required for the chitosan gelation (**Reaction 1**) was produced directly *in-situ* thanks to the urea hydrolysis by urease enzyme (**Reaction 2**) and the equilibrium of ammonia/ammonium (**Reaction 3**).



This chitosan enzymatic gelation has been further experimentally investigated and used to produce chitosan hydrogel for different applications such as delivery periodontal ligament cells or membrane preparation [14 - 16]. The gelation time of chitosan mainly depends on urease concentration [14, 16] and temperature [16] while urea concentration was shown to have nearly no influence if this concentration is high enough to prevent urea depletion before the gelation [15, 16]. In the aforementioned studies, the gelation kinetics have been monitored by pH measurements [15, 16] and compared in both aqueous and chitosan media. The main conclusions of those studies were: (i) presence of chitosan seemed to have a weak influence

on enzyme activity during urea hydrolysis [16] and (ii) the difference may only be related to the chitosan amino-group equilibrium. However, this assumption has not yet been confirmed in these previous studies. Moreover, pH measurement only allows a partial understanding of the global chitosan enzymatic gelation since kinetics of simultaneous chemical reaction involved in chitosan gelation (Reaction 1) and urea enzymatic hydrolysis (Reaction 2) cannot be dissociated by a pH monitoring only. Investigating and modeling the kinetics of urea hydrolysis reaction in chitosan medium is therefore a key point to deeply understand the global chitosan enzymatic gelation and to predict chitosan gelation time. In this context, the influence of various operating parameters (temperature, urease concentration, urea concentration, pH, product inhibition, substrate inhibition) on urea enzymatic hydrolysis kinetics is well known in aqueous medium [17] and these urea hydrolysis kinetics have been successfully modeled for pH-buffered aqueous medium [18] and buffer-free aqueous medium [19] but, to our knowledge, the same investigation has never been conducted in chitosan solution.

Based on prior urea hydrolysis kinetics modeling in aqueous medium, the aim of this paper is to develop a model of both urea hydrolysis in chitosan medium and global enzymatic chitosan gelation. Modeling and experimental results of enzymatic chitosan gelation were compared to validate the numerical model. Modeling results in aqueous medium and in chitosan solution was compared in order to understand the influence of chitosan presence on the urea hydrolysis reaction. Finally, the chitosan gelation and urea hydrolysis kinetics were dissociated and investigated.

2. Materials and methods

2.1 Materials

Chitosan (obtained from France Chitine, France) had a degree of deacetylation (**DDA**) of 80% (supplier's data determined by nuclear magnetic resonance) and a weight-average molecular weight (MW_{chit}) of 180,000 g.mol⁻¹ (supplier's data determined by size-exclusion chromatography). Urea, urease and hydrochloric acid were purchased from Sigma (France). Urease (type III, EC 3.5.1.5) from *Canavalia ensiformis* (Jack bean) was supplied as a glycerol solution and the specific activity of this enzymatic solution was 778,000 U.L⁻¹ [16].

2.2 Enzymatic urea hydrolysis in buffer-free chitosan solution and in buffer-free water

medium

Enzymatic urea hydrolysis in buffer-free chitosan solution and in buffer-free water medium was carried out in a 10 mL glass batch reactor according to the protocol defined in a previous study [16]. Osmotic water medium and chitosan solution (2.5 % w/v) were prepared using *HCl* 0.1 mol.L⁻¹ in order to obtain an initial buffer-free pH of 4.7 ± 0.1. Reactions were performed at different temperatures (274 to 310 K), urea concentrations (0.010 to 0.200 mol.L⁻¹) and urease concentrations (500 ± 10 to 10,500 ± 10 U.L⁻¹). Reactions were monitored by pH measurements using a Hanna Instruments pH-meter (HI 2214 pH/ORB) and a specific probe for viscous media (HI 2031, Hanna Instruments) in both chitosan solution and water media. For each assay, pH was continuously recorded every 5 seconds until the pH reached a plateau [16]. A reference experiment in chitosan solution was reproduced at least five times to determine the uncertainty on pH (± 0.26) [16].

3. Modeling of the chitosan enzymatic gelation kinetics

The mathematical modeling of the chitosan gelation kinetics can be divided in three parts: the chemical equilibrium reaction involved in chitosan gelation (Section 3.1), the urea enzymatic hydrolysis by enzyme urease (Section 3.2) and the chemical equilibrium reactions of the other species in solution (Section 3.3). The modeling algorithm will be described in Section 3.4.

Species are considered in dilute aqueous solutions and activities of the species are assumed to be equal to their concentrations. The activity of the solvent (water) is taken equal to 1. In this study, the whole reactions were modeled in conditions of batch and perfectly mixed reactor.

3.1 Chemical equilibrium reaction involved in chitosan gelation

Chitosan gelation results from the chitosan chemical equilibrium reaction (Reaction 1) between the protoned **Chit** – **NH₃⁺** and non-protoned **Chit** – **NH₂** forms of the amino-groups of the chitosan *D-glucosamine* units. Mass balance on these amino-groups is given at each time *t* in Equation 1 with the total molar concentration [**Chit** – **N**]₀ of amino-groups in solution (mol.L⁻¹), the total molar concentration [**Chit** – **NH₃⁺**] of protoned amino-groups in solution (mol.L⁻¹) and the total molar concentration [**Chit** – **NH₂**] of non-protoned amino-groups in solution (mol.L⁻¹).

$$[\mathbf{Chit} - \mathbf{N}]_0 = [\mathbf{Chit} - \mathbf{NH}_3^+] + [\mathbf{Chit} - \mathbf{NH}_2] \quad \text{Equation 1}$$

The pKa of these amino-groups of chitosan ranged between 6.2 and 7.2 [6, 7] and depends on the chitosan degree of deacetylation. A mean value of 6.7 was retained in this study. The expression of the related equilibrium constant K_1 is given with Equation 2.

$$K_1 = \frac{(\text{Chit-NH}_2)(\text{H}_3\text{O}^+)}{(\text{Chit-NH}_3^+)(\text{H}_2\text{O})} = \frac{[\text{Chit-NH}_2][\text{H}_3\text{O}^+]}{[\text{Chit-NH}_3^+] \times 1} = 10^{-6.7} \quad \text{Equation 2}$$

Chitosan is characterized by its degree of deacetylation DDA (%) and its weight-average molecular weight MW_{Chit} (g.mol⁻¹). The mean molecular weight MW_{Glc} (g.mol⁻¹) of an unit of the chitosan chain can be calculated according to Equation 3 with the *D-glucosamine* unit molecular weight MW_{GlcN} (179.17 g.mol⁻¹) and the *N-acetyl-glucosamine* unit molecular weight MW_{GlcNAc} (221.21 g.mol⁻¹). (Figure 1)

$$MW_{\text{Glc}} = \left(\frac{DDA}{100}\right) \times MW_{\text{GlcN}} + \left(\frac{100-DDA}{100}\right) \times MW_{\text{GlcNAc}} \quad \text{Equation 3}$$

The number N_{GlcN} (mol.mol⁻¹) of moles of *D-glucosamine* by mole of chitosan can be deduced from Equation 4.

$$N_{\text{GlcN}} = \left(\frac{DDA}{100}\right) \times \frac{MW_{\text{Chit}}}{MW_{\text{Glc}}} \quad \text{Equation 4}$$

If chitosan is initially in solution at a mass concentration C_C (% w/v) or C_C' (g.L⁻¹), the concentration of *D-glucosamine* amino-groups in solution can be calculated with Equation 5.

$$[\text{Chit} - \text{N}]_0 = \frac{C_C'}{MW_{\text{Chit}}} \times N_{\text{GlcN}} = \frac{10 \times C_C}{MW_{\text{Chit}}} \times N_{\text{GlcN}} \quad \text{Equation 5}$$

3.2 Urea enzymatic hydrolysis reaction

Reaction of urea enzymatic hydrolysis by urease is given in Reaction 2 and produced both ammonia and carbon dioxide. The rate of this reaction r_{hydro} (mol.L⁻¹.min⁻¹) is defined as the consumption rate of urea [(NH₂)₂CO] (mol.L⁻¹) according to Equation 6.

$$r_{\text{hydro}} = - \frac{d([(NH_2)_2CO])}{dt} \quad \text{Equation 6}$$

Considering an uncompetitive substrate inhibition (by urea at high concentration) and a non-competitive mechanism for product inhibition (ammonium resulting from the ammonia/ammonium equilibrium in Reaction 3), urea hydrolysis by urease from *Canavalia ensiformis* (Jack bean) has been modeled for aqueous buffered or buffer-free medium using a

similar expression (Equation 7) of hydrolysis reaction rate r_{hydro} . So, the influence of temperature, pH, urea concentration, urease concentration and ammonium concentration on the urea hydrolysis reaction rate was taken into consideration [18-19].

$$r_{hydro} = \frac{v_{max}[(NH_2)_2CO]}{\left(K_M + [(NH_2)_2CO] + \frac{[(NH_2)_2CO]^2}{K_S}\right) \left(1 + \frac{[NH_4^+]}{K_P}\right)} = \frac{k_{T,pH}[E]_0[(NH_2)_2CO]}{\left(K_M + [(NH_2)_2CO] + \frac{[(NH_2)_2CO]^2}{K_S}\right) \left(1 + \frac{[NH_4^+]}{K_P}\right)} \quad \text{Equation 7}$$

with the Michaelis constant K_M (mol.L⁻¹), the substrate inhibition constant K_S (mol.L⁻¹), the product inhibition constant K_P (mol.L⁻¹) and the enzymatic maximum reaction rate v_{max} (mol.L⁻¹.min⁻¹) that can be expressed using the temperature and pH-dependent hydrolysis reaction rate coefficient $k_{T,pH}$ (mol.U⁻¹.min⁻¹) and the urease concentration $[E]_0$ (U.L⁻¹). Unlike previous modeling in water medium, it was preferred in the present study to express the urease concentration in U.L⁻¹ instead of g.L⁻¹ (where one catalytic unit (U) is defined as the enzyme quantity that will liberate 1.0 μmol of NH₃ from urea per liter and per minute at pH 7.0 and 298 K) in order to take into consideration a possible loss of enzyme activity.

Hydrolysis reaction rate coefficient $k_{T,pH}$ depends on temperature and pH and can be expressed according to Equation 8 [18, 19] with the hydrolysis temperature-dependent reaction rate constant k_T (mol.U⁻¹.min⁻¹) and the pH-dependent correction factor ϕ_{pH} (-).

$$k_{T,pH} = \phi_{pH} k_T = \frac{k_0 \exp\left(\frac{-Ea}{RT}\right)}{\left\{1 + \left(\frac{10^{-pH}}{K_{ES,1}}\right)^{\alpha_{ES,1}} + \left(\frac{K_{ES,2}}{10^{-pH}}\right)^{\alpha_{ES,2}}\right\}} \quad \text{Equation 8}$$

Temperature-dependent reaction rate coefficient k_T was supposed to follow Arrhenius Equation [19] with the Arrhenius Equation pre-exponential factor k_0 (mol.L⁻¹.U⁻¹.min⁻¹), the activation energy Ea (J.mol⁻¹) and the universal gas constant R (J.mol⁻¹.K⁻¹) as shown in Equation 9.

$$k_T = k_0 \exp\left(\frac{-Ea}{RT}\right) \quad \text{Equation 9}$$

pH-dependent correction factor ϕ_{pH} can be expressed according to Equation 10 [18] where $K_{ES,1}$ and $K_{ES,2}$ are the molecular dissociation constants for the enzyme-substrate complex and $\alpha_{ES,1}$ and $\alpha_{ES,2}$ are empirical temperature-independent constants.

$$\phi_{pH} = \frac{1}{\left\{1 + \left(\frac{10^{-pH}}{K_{ES,1}}\right)^{\alpha_{ES,1}} + \left(\frac{K_{ES,2}}{10^{-pH}}\right)^{\alpha_{ES,2}}\right\}} \quad \text{Equation 10}$$

Finally, according to Reaction 2, production rate of ammonia concentration $[NH_3]$ (mol.L⁻¹) and carbon dioxide concentration $[CO_2]$ (mol.L⁻¹) can be expressed following Equations 11 and 12.

$$\frac{d([NH_3])}{dt} = 2r_{hydro} \quad \text{Equation 11}$$

$$\frac{d([CO_2])}{dt} = r_{hydro} \quad \text{Equation 12}$$

3.3 Chemical equilibrium reactions in aqueous solution

Since urea enzymatic hydrolysis produces ammonia and carbon dioxide (Reaction 2), two other reaction equilibria have to be taken into account: the ammonium/ammonia equilibrium reaction (Reaction 3') and the carbon dioxide/hydrogen carbonate/carbonate equilibrium reactions (Reactions 4 and Reaction 5).



Equations and constants of these equilibria are presented in Table 1 (Equations 13, 14, 15) with also the hydronium-hydroxide equilibrium (Equation 16) using the ammonium $[NH_4^+]$ (mol.L⁻¹), carbonate $[CO_3^{2-}]$ (mol.L⁻¹), hydrogen carbonate $[HCO_3^-]$ (mol.L⁻¹), hydroxide $[OH^-]$ (mol.L⁻¹) and the hydronium $[H_3O^+]$ (mol.L⁻¹) concentrations. The hydronium concentration can be expressed with the pH according to Equation 17 and the water dissociation constant K_W (-) is reported in Table 1. Ammonium/ammonia equilibrium constant K_3 is considered temperature-independent while the influence of the temperature T (K) is considered for the carbon dioxide/hydrogen carbonate/carbonate equilibrium constants K_4 and K_5 .

$$[H_3O^+] = 10^{-pH} \quad \text{Equation 17}$$

Table 1: Equilibrium equations and constants in aqueous solutions

<i>Equilibrium</i>	<i>Equations</i>	<i>Constant values</i>	<i>Ref.</i>
$\text{NH}_4^+ / \text{NH}_3$	$K_3 = \frac{(\text{NH}_3)(\text{H}_3\text{O}^+)}{(\text{NH}_4^+)(\text{H}_2\text{O})} = \frac{[\text{NH}_3][\text{H}_3\text{O}^+]}{[\text{NH}_4^+] \times 1}$ <p style="text-align: center;"><i>Equation 13</i></p>	$10^{-9.3}$ (at 298 K)	[20]
$\text{HCO}_3^- / \text{CO}_3^{2-}$	$K_4 = \frac{(\text{CO}_3^{2-})(\text{H}_3\text{O}^+)}{(\text{HCO}_3^-)(\text{H}_2\text{O})} = \frac{[\text{CO}_3^{2-}][\text{H}_3\text{O}^+]}{[\text{HCO}_3^-] \times 1}$ <p style="text-align: center;"><i>Equation 14</i></p>	$7.83 \times 10^{-8} e^{-2213/T}$	[21]
$\text{CO}_2 / \text{HCO}_3^-$	$K_5 = \frac{(\text{HCO}_3^-)(\text{H}_3\text{O}^+)}{(\text{CO}_2)(\text{H}_2\text{O})^2} = \frac{[\text{H}_3\text{O}^+][\text{HCO}_3^-]}{[\text{CO}_2] \times 1^2}$ <p style="text-align: center;"><i>Equation 15</i></p>	$5.02 \times 10^{-5} e^{-1462.7/T}$	[21]
$\text{HO}^- / \text{H}_3\text{O}^+$	$K_W = (\text{H}_3\text{O}^+) \times (\text{OH}^-) = [\text{H}_3\text{O}^+] \times [\text{OH}^-]$ <p style="text-align: center;"><i>Equation 16</i></p>	10^{-14}	[20]

Following ammonium/ammonia equilibrium and carbon dioxide/hydrogen carbonate/carbonate equilibrium, Equations 11 and 12 can be rewritten as Equations 18 and 19, respectively.

$$\frac{d([\text{NH}_4^+] + [\text{NH}_3])}{dt} = 2r_{\text{hydro}} = -2 \frac{d([\text{(NH}_2)_2\text{CO}])}{dt} \quad \text{Equation 18}$$

$$\frac{d([\text{CO}_3^{2-}] + [\text{HCO}_3^-] + [\text{CO}_2])}{dt} = r_{\text{hydro}} = - \frac{d([\text{(NH}_2)_2\text{CO}])}{dt} \quad \text{Equation 19}$$

The initial concentrations of ammonium, ammonia, carbon dioxide, hydrogen carbonate and carbonate are considered null and, as no external transfer is considered, the mass balance for ammonium/ammonia equilibrium and dioxide/hydrogen carbonate/carbonate equilibrium can be written at each time t (min) according to Equation 20 and 21 where $[(\text{NH}_2)_2\text{CO}]_0$ (mol.L⁻¹) is the initial urea concentration:

$$[\text{NH}_4^+] + [\text{NH}_3] = 2([\text{(NH}_2)_2\text{CO}]_0 - [(\text{NH}_2)_2\text{CO}]) \quad \text{Equation 20}$$

$$[\text{CO}_3^{2-}] + [\text{HCO}_3^-] + [\text{CO}_2] = [(\text{NH}_2)_2\text{CO}]_0 - [(\text{NH}_2)_2\text{CO}] \quad \text{Equation 21}$$

Finally, the electronic balance is given in Equation 22 with the chloride concentration $[\text{Cl}^-]$ (mol.L⁻¹).

$$[\text{NH}_4^+] + [\text{H}_3\text{O}^+] + [\text{Chit} - \text{NH}_3^+] = 2[\text{CO}_3^{2-}] + [\text{OH}^-] + [\text{HCO}_3^-] + [\text{Cl}^-] \quad \text{Equation 22}$$

3.4 Modeling numerical resolution

Figure 2 presents a scheme of the resolution strategy. Initial parameters, *i.e.* at the beginning of the chitosan enzymatic gelation (for time $t = 0$ min), are the temperature T (K), $\mathbf{pH} = \mathbf{pH}_0$, the urease concentration $[E]_0$ (U.L⁻¹), the urea initial concentration $[(NH_2)_2CO] = [(NH_2)_2CO]_0$ (mol.L⁻¹), chloride concentration $[Cl^-]$ (mol.L⁻¹) and the chitosan mass concentration C_C (% w/v) that allowed calculating the concentration $[Chit - N]_0$ of *D-glucosamine* amino-groups in solution (Equation 3, 4, 5). Temperature, urease concentration and chitosan mass concentration were verified to remain constant during all chitosan enzymatic gelation while pH and urea concentration are time-dependent variables. All other species, except obviously hydroxide and hydronium, have an initial concentration equal to zero.

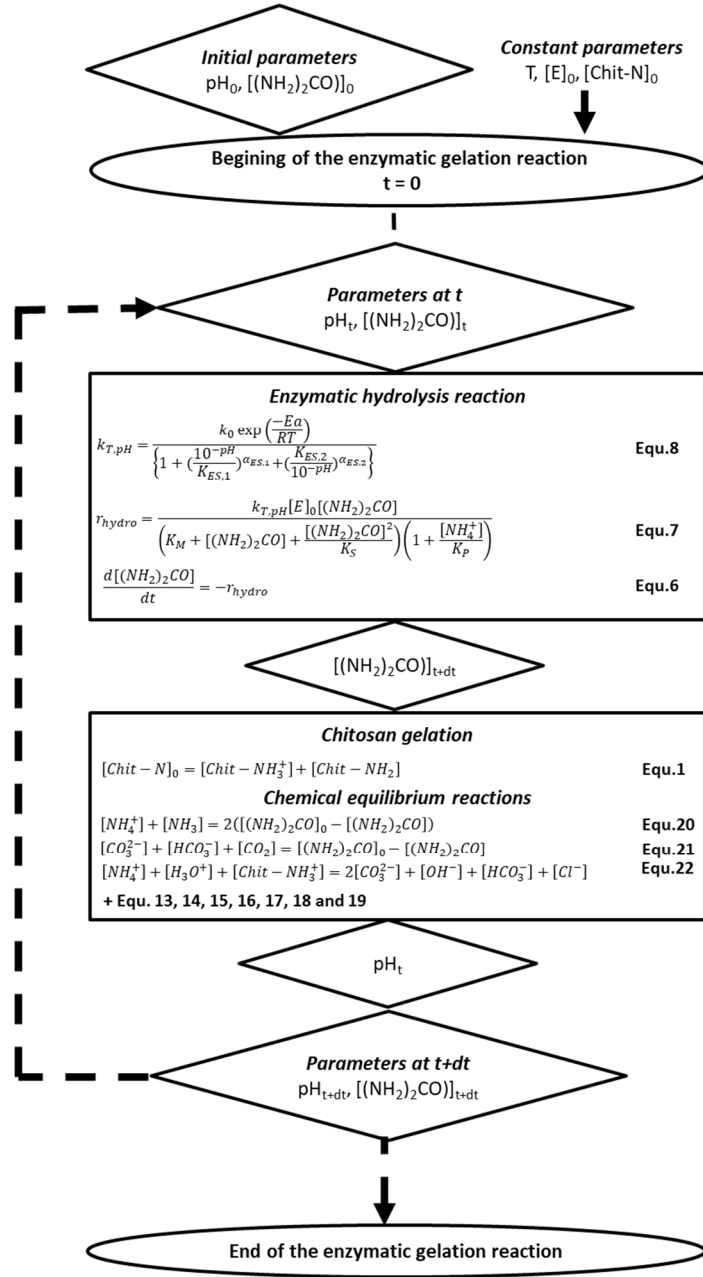


Figure 2: Modeling numerical resolution strategy

Numerical time-resolution of the modeling was performed using finite difference method with MATLAB-software. Time-step $dt = 0.5 \text{ min}$ (30 s) was found to be a good compromise between the resolution time and results precision for modeling in chitosan medium while a time-step $dt = 0.001 \text{ min}$ (0.06 s) was required for modeling in water medium due to faster reactions involved. For each time-step, simulation resolution was performed in two stages. The first stage was the enzymatic hydrolysis reaction: the urea hydrolysis rate was calculated using Equations 7 and 8 at the pH and urea concentration at the time t . Urea concentration, total ammonia/ammonium concentration and total carbon dioxide/hydrogen

carbonate/carbonate concentration were thus calculated at time $t + dt$ using Equations 6,18 and 19 by finite difference method. The second stage was the calculation of the chitosan gelation and chemical aqueous equilibrium reactions. The set of Equations 1, 2, 13, 14, 15, 16, 17, 20, 21 and 22 was solved using build-in fsolve MATLAB® software function (least square method) to calculate hydronium concentration and pH at the time $t + dt$. Concentrations of the other species were calculated at this time $t + dt$ using the same set of equations. This routine of two stages was performed for each time-step until the end of the reaction.

4. Results

4.1 Determination of the kinetics parameters for the reaction involved in enzymatic gelation

Enzymatic hydrolysis reaction rate r_{hydro} reaction depends on several parameters, the Michaelis constant K_M , the ammonium enzyme inhibition constant K_P , the urea enzyme inhibition constant K_S and the hydrolysis reaction rate coefficient $k_{T,pH}$ which depends itself on the molecular dissociation constant for the enzyme-substrate complexes $K_{ES,1}$ and $K_{ES,2}$ (and their respective associated empirical factor $\alpha_{ES,1}$ and $\alpha_{ES,2}$), the Arrhenius Equation pre-exponential factor k_0 and the activation energy Ea . Chitosan may have an influence on these parameters and they have to be determined in chitosan medium using specific experimental results: two sets of experiments were thus used for a 2.45% w/v chitosan concentration, a 3,500 U.L⁻¹ urease concentration and an initial pH equal to 4.7 ± 0.1 :

(i) A first set of experiments corresponding to an urea concentration of 0.075 mol.L⁻¹ and for 274.15, 278.15, 283.15, 288.15, 293.15, 298.15, 303.15 and 310.15 K temperatures. Experiments at 274.15 K and 298.15 K are from the present study while others are from [16].

(ii) A second set of experiments for a temperature of 298.15K and 0.075, 0.100, 0.150 and 0.200 mol.L⁻¹ for the urea concentration. Experiment at 0.200 mol.L⁻¹ is from the present study while others are from [16].

For each experiment, data of pH *versus* time were used. At each reaction time, urea concentration $[(NH_2)_2CO]$ was calculated from pH using the chitosan gelation and chemical equilibrium reactions, *i.e.* the set of Equations 1, 2, 13, 14, 15, 16, 17, 20, 21 and 22 using build-in fsolve MATLAB®software function (least square method). Experimental hydrolysis reaction rate r_{exp} (mol.L⁻¹.min⁻¹) was then calculated using Equation 23:

$$r_{exp} = - \frac{d[(NH_2)_2CO]}{dt}$$

Equation 23

The values of the enzymatic hydrolysis reaction rate parameters for this study were determined using experimental data and reported in Table 2. This table also presents, for comparison, parameters from literature for both buffer-free water medium [19] and buffered water medium [18], *i.e.* without chitosan. In this table, urease mass (g) has been converted in urease activity (U) using the enzyme activity-mass conversion factor $k' = 1.15 \times 10^4 \text{ U.g}^{-1}$ determined from supplier data for the enzyme used in the present study. Furthermore, k_0 was calculated from T^* (*i.e.* the temperature in K at which $k_T = 1 \text{ mol.g}^{-1} \cdot \text{min}^{-1}$) and Ea for [18].

Table 2: Enzymatic reaction kinetics validation range and parameters

	Source	This study	[19]	[18]
Validation range	Urease concentration (U.L ⁻¹)	500 – 10,500	4.6 – 115	230 – 2,300
	Urea concentration (mol.L ⁻¹)	0 – 0.200	0 – 3	0 – 0.070
	pH	4 – 9 (Buffer-free)	5 – 9 (Buffer-free)	4 – 9 (Buffered)
	Ammonium (mol.L ⁻¹)	0 – 0.400	0 – 0.040	0 – 0.080
	Temperature (K)	274 – 310	293 - 313	298 – 310
	Chitosan concentration (% w/v)	2 – 4	0	0
Modeling parameters	K_M (mol.L ⁻¹)	$(3.21 \pm 0.01) \times 10^{-3}$	2.56×10^{-3}	$(3.21 \pm 0.36) \times 10^{-3}$
	K_P (mol.L ⁻¹)	$+\infty$	6.93×10^{-2}	$(1.22 \pm 0.11) \times 10^{-2}$
	K_S (mol.L ⁻¹)	$+\infty$	6.18	$+\infty$
	Ea (J.mol ⁻¹)	22,221 \pm 1	29,100	35,300
	T^* (K)	-	-	414.6
	k_0 (mol.U ⁻¹ .min ⁻¹)	$(4.14 \pm 0.01) \times 10^{-3}$	6.96×10^{-4}	2.44
	$K_{ES,1}$ (mol.L ⁻¹)	$(1.04 \pm 0.01) \times 10^{-6}$	$10^{-5.62}$ (298 K), $10^{-5.28}$ (308 K), $10^{-5.15}$ (318 K)	$(7.57 \pm 0.04) \times 10^{-7}$
	$K_{ES,2}$ (mol.L ⁻¹)	$(2.98 \pm 0.01) \times 10^{-9}$	$10^{-9.07}$ (298 K), $10^{-9.43}$ (308 K), $10^{-9.58}$ (318 K)	$(1.27 \pm 0.08) \times 10^{-8}$
	$\alpha_{ES,1}$	1	0.564 (298 K), 0.717 (318 K), 0.769 (318 K)	1
	$\alpha_{ES,2}$	1	0.373 (298 K), 0.489 (308 K), 0.542 (318 K)	1

Michaelis constant K_M has been reported to be ranged between 2.9×10^{-3} and 3.6×10^{-3} mol.L⁻¹ according to the purity of the enzyme and the conditions for its determination like temperature, buffered or buffer-free conditions, pH and concentration [17]. An intermediate value of 3.21×10^{-3} mol.L⁻¹ was retained for the present work (Table 2) and it was considered independent of the temperature and pH [18].

An uncompetitive inhibition mechanism of urease by substrate urea can be generally considered and values of urea inhibition constant K_S from 3 to 6.4 mol.L⁻¹ have been reported

in literature [17, 19, 22]. In the same way, a non-competitive inhibition mechanism of urease by product ammonium have been reported in literature with inhibition constant K_P from 2×10^{-3} to 118×10^{-3} mol.L⁻¹ [17, 18, 23]. Due to the small urea and ammonium concentrations in this study, these inhibitions were neglected and both K_S and K_P were thus considered infinite.

The temperature-dependent reaction coefficient k_T was used as fitting parameter, at each reaction time, between modeling value of hydrolysis rate r_{hydro} (Equation 8) and experimental value of hydrolysis rate r_{exp} (Equations 23) using least square minimization for the results of the first set of experiments (temperature between 274.15 and 310.15K). An Arrhenius pre-exponential factor k_0 of 4.14×10^{-3} mol.U⁻¹.min⁻¹ was found, which is higher than the one reported by Qin *et al.* 1994 [19] but the comparison is difficult since the mass/activity conversion factor k' was established for the present work though it should be different in their work. Activation energy E_a of 22,221 J.mol⁻¹ was found (Table 2), which is slightly lower than results reported in literature for buffer-free medium (29,440 J.mol⁻¹ [19] or 27,781 J.mol⁻¹ [24]). This might be due to the larger temperature range used in the present work (a temperature range of 293.15 to 313.15 K have been generally used in other studies).

The pH correction factor ϕ_{pH} was considered temperature independent as reported by Fidaleo *et al.* 2003 [18] and unlike Qin *et al.* 1994 [19]. At each reaction time, ϕ_{pH} was used as fitting parameter between modeling value r_{hydro} (Equation 8) and experimental value r_{exp} (Equations 23) using least square minimization for results of the second set of experiment. Considering $\alpha_{ES,1} = \alpha_{ES,2} = 1$, values for the molecular dissociation constant were found to be $K_{ES,1} = (1.04 \pm 0.01) \times 10^{-6}$ and $K_{ES,2} = (2.98 \pm 0.01) \times 10^{-9}$ mol.L⁻¹.

4.2 Comparison between modeling and experimental results in chitosan aqueous solution

The simulation results of the enzymatic chitosan gelation were compared to experimental results from our previous work [16] for a buffer-free 2.5% w/v chitosan solution at initial pH = 4.7. Figure 3 presents pH time-variation during the chitosan enzymatic gelation [16]: i) for different urea initial concentrations $[(NH_2)_2CO]_0$ with a temperature of 298 K and an urease concentration of 3,500 U.L⁻¹ (Figure 3a); ii) for different values of the temperature T with an urea initial concentration of 0.075 mol.L⁻¹ and an urease concentration of 3,500 U.L⁻¹ (Figure 3b); iii) and for different urease concentrations $[E]_0$ with a temperature of 298 K and

an urea initial concentration of 0.075 mol.L^{-1} (Figure 3c).

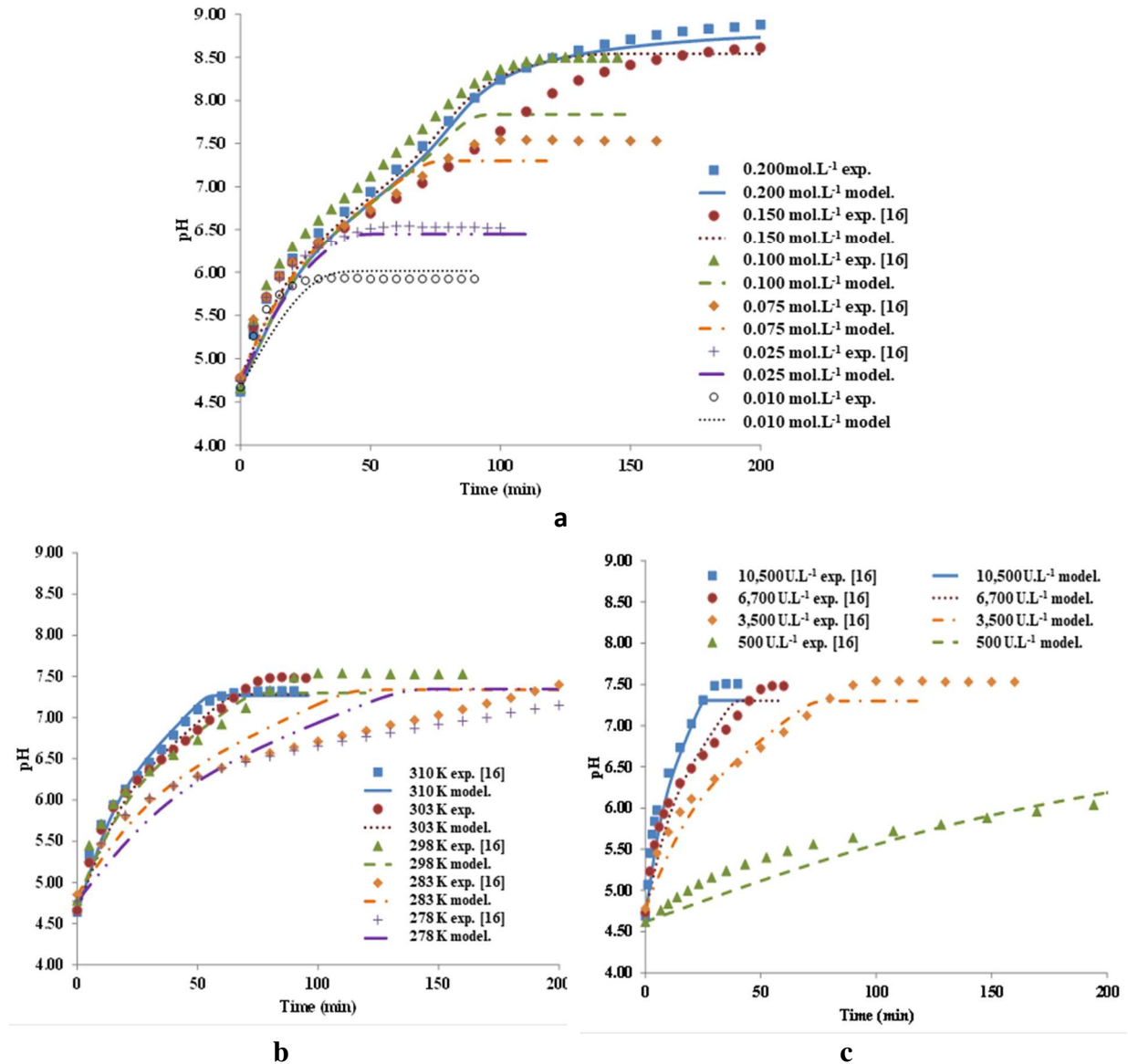


Figure 3: Comparison of experimental and modeling pH variations during chitosan enzymatic gelation for a buffer-free 2.5% w/v chitosan solution of a) assay of initial urea concentration with $T = 298 \text{ K}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$; b) assay of temperature with $[(\text{NH}_2)_2\text{CO}]_0 = 0.075 \text{ mol.L}^{-1}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$; and c) assay of urease concentration with $T = 298 \text{ K}$ and $[(\text{NH}_2)_2\text{CO}]_0 = 0.075 \text{ mol.L}^{-1}$

Figure 3 shows a fairly good concordance between experimental and modeling results, thus validating the model and allowing the determination of its associated kinetics parameters (Table 2) for a large range of operating conditions: an urease concentration $[E]_0$ between 500 and $10,500 \text{ U.L}^{-1}$, an urea concentration between 0 and 0.200 mol.L^{-1} , a pH between 4.0 and 9.0 and a temperature between 274 and 310 K. However, some differences can be still raised

between modeling and experimental data that should be investigated more deeply.

First, except for the experiments where urea is a limiting parameter due to its too low concentration (below 0.050 mol.L^{-1}), the modeling results always underestimate the plateau of pH at the end of the experiments (Figure 3a). This plateau indicates the end of the experiment, *i.e.* the depletion of urea. It would indicate that the depletion of urea occurred later and that there was initially more urea in the solution than expected (which allows reaching a higher pH value). In Section 4.1, urea concentration during the reaction, used for the determination of kinetics parameters of the reaction involved in enzymatic gelation, was estimated using pH value. However, uncertainty may exist on chitosan characteristics such as the pKa of amino-groups, the degree of deacetylation and the molecular weight used for the calculation of the amino-group concentration which thus could induce slight uncertainty on urea concentration.

Secondly, modeling and experimental results are less concordant at low temperatures (Figure 3b) like 283 K or less. It might be related to a divergence from Arrhenius Equation at such low temperatures. Indeed, in water medium, enzyme activity has only been modeled for temperature higher than 293 K (Table 2) and a significant decrease of enzymatic activity has been considered in some studies for temperature lower than 278 K [13].

Finally, modeling and experimental results diverge for intermediate pH values (around 7-8) in some experiments (for example experiment at an urea concentration of 0.150 mol.L^{-1} in Figure 3a). This phenomenon was also observed in water medium [19]. A possible explanation could be a transfer of CO_2 at the interface between the solution and the external environment around this pH, and thus experimental pH could be higher than predicted by the modeling. Another explanation is the possible inhibition by the product ammonium that would no longer be negligible. Indeed, around these intermediate pH values, ammonium concentration tends to be higher while urea concentration is still high and hence is not yet a limiting factor.

Even if slight differences may be found between the modeling and experimental results of the pH variations *versus* time, these uncertainties have only a weak influence on the prediction of the gelation time t_g and the advanced gelation time t_{ag} . Gelation time is the time when chitosan elastic modulus G' and its viscous modulus G'' are equals in rheological measurements [15, 16, 25]. The advanced gelation time is the time when a completely rigid gel is obtained [16]. From our previous work [16], gelation time corresponded to pH 6.5 while

advanced gelation time corresponded to pH 7.35. Figure 4 presents a comparison between experimental and predicted values of gelation time and advanced gelation time (when the experiment allows reaching its value).

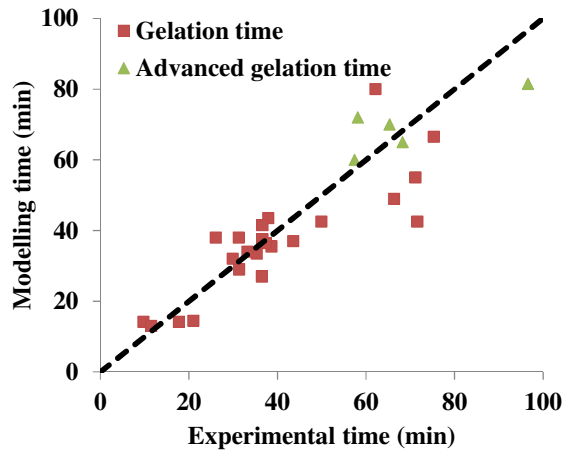


Figure 4: Comparison of the experimental and modeling gelation and advanced gelation times

Figure 4 shows a very good agreement between predicted and experimental characteristic times. An average difference of 20% is observed for the gelation time and of 16% for the advanced gelation time. Enzymatic chitosan gelation modeling can thus be a good tool to predict gelation and advanced gelation time.

4.3 Urea enzymatic hydrolysis reaction kinetics in water and chitosan media

One of the aims of the enzymatic chitosan gelation modeling was to determine the possible influence of chitosan on the urea enzymatic hydrolysis kinetics. The same enzymatic chitosan gelation modeling was applied in water medium (*i.e.* for a chitosan concentration C_c equal zero) without modifying the enzyme and kinetic parameters determined in Section 4.1 for chitosan medium. Figure 5 presents experimental [16] and modelling pH time-variation during urea enzymatic hydrolysis in non-buffered aqueous medium at initial pH 4.7 for different urea initial concentrations $[(NH_2)_2CO]_0$ with a temperature of 298 K and an urease concentration of 500 U.L⁻¹ (Figure 5a); and for different temperatures T with an urea initial concentration of 0.075 mol.L⁻¹ and an urease concentration of 500 U.L⁻¹ (Figure 5b). It must be noted that an urease concentration of 500 U.L⁻¹ was used here (instead of 3,500 U.L⁻¹ in chitosan medium) in order to slow down pH increase, hence to visualize an effect of the operating conditions.

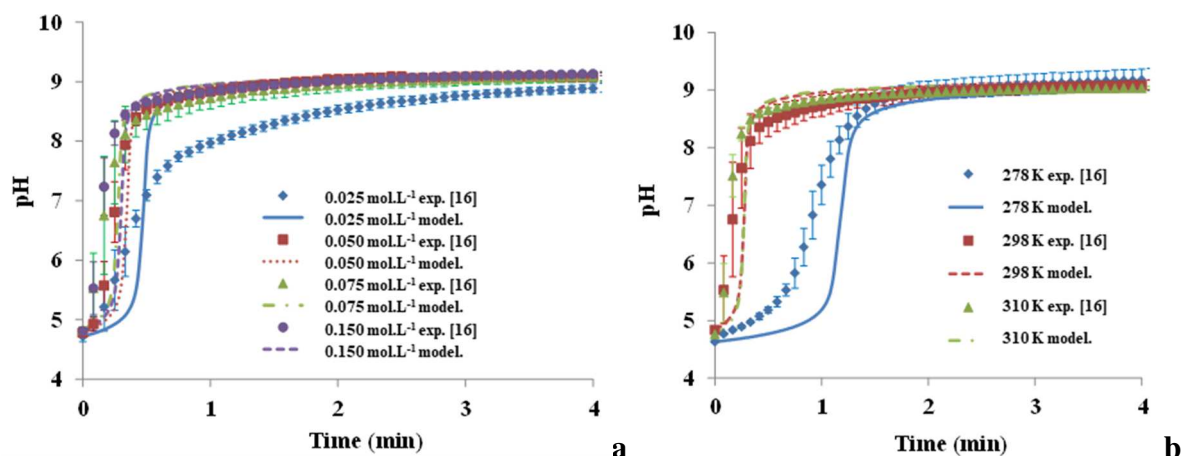


Figure 5: Comparison of experimental and modeling pH variations during enzymatic urea hydrolysis for non-buffered aqueous medium for $[E]_0 = 500 \text{ U.L}^{-1}$: a) influence of $[(\text{NH}_2)_2\text{CO}]_0$ at $T = 298 \text{ K}$ and b) influence of T with $[(\text{NH}_2)_2\text{CO}]_0 = 0.075 \text{ mol.L}^{-1}$

Important differences concerning the pH time-variations were observed between water medium and chitosan medium [16]: for example a pH 6.5 was reached in less than one minutes for an urea concentration of 0.075 mol.L^{-1} , a temperature of 298 K and an urease concentration of 500 U.L^{-1} in water medium (Figure 5a) while a pH of nearly 6 was only reached after 200 minutes in chitosan medium. In water medium, pH increased very fast before reaching an “apparent” plateau around 8.5 – 9. This fast increase of pH can be directly related to the ammonia produced by urea hydrolysis reaction (Reactions 2 and 3) and thus to the urea enzymatic hydrolysis reaction kinetics.

In chitosan medium, pH increased more slowly but reached a plateau as well. However, in this case the pH could no more be only related to the ammonia produced by urea hydrolysis reaction (Reactions 2 and 3) because of the presence of the chitosan reaction equilibrium (Reaction 1). pH variation is related to the reaction kinetics of the global enzymatic chitosan gelation (Reactions 1, 2 and 3). The chitosan equilibrium reaction is thus the most suitable explanation to understand why the variation of pH is so different between water and chitosan media. Indeed, a good concordance between modeling and experimental results was observed in water medium using enzyme and kinetics parameters established for chitosan medium.

At last, it was interesting to focus on the difference between modeling and experimental results when urea concentration is considered a limiting factor (for 0.025 mol.L^{-1} in Figure 5a) while a very good concordance was observed in chitosan medium in the same condition

(Figure 3a). The difference could be related to a possible product inhibition and may be more visible for low initial urea concentration when there is no product consumption unlike in the case of chitosan medium.

In both chitosan medium and water medium, pH seemed to reach a plateau at the end of the reaction. However, explanations of this behaviour are totally different for chitosan and water media since a “real” plateau was obtained in chitosan medium though only an “apparent” plateau was reached in water medium. Figure 6 presents simulated urea concentration *versus* time for chitosan medium (Figure 6a, experiments from Figure 3a) and for water medium (Figure 6b, experiments from Figure 5a)

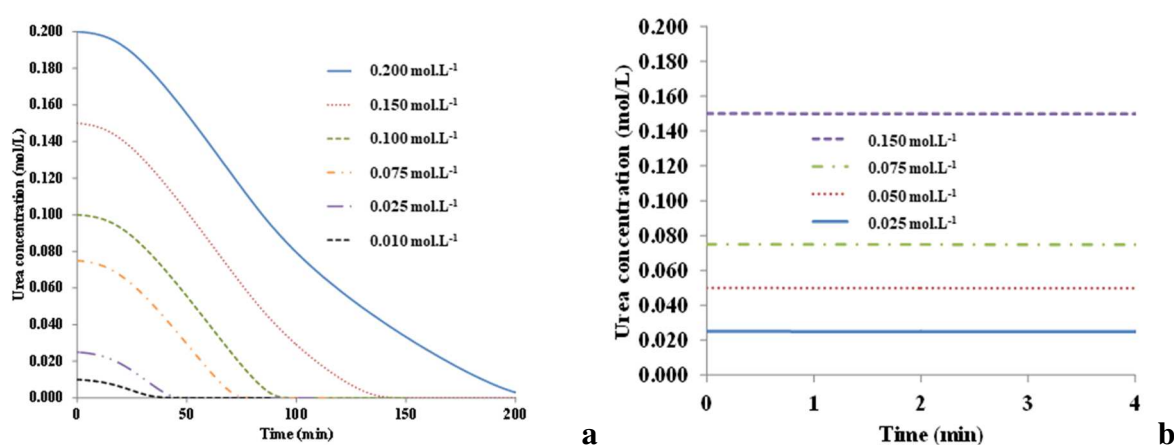


Figure 6: Variation of urea concentration *versus* time for different urea initial concentration at T = 298 K during a) chitosan enzymatic gelation for a buffer-free 2.5% w/v chitosan solution with $[E]_0 = 3,500 \text{ U.L}^{-1}$ and b) during enzymatic urea hydrolysis for a buffer-free aqueous medium with $[E]_0 = 500 \text{ U.L}^{-1}$

Figure 6a shows that urea concentration dropped to zero at the end of the urea enzymatic hydrolysis reaction in chitosan medium. This depletion of urea occurred when the pH reached a “real” plateau (Figure 3a). The level of this plateau, *i.e.* the final value of pH, is only related to the final cumulated production of the urea hydrolysis reaction and thus to the urea initial concentration (higher pH plateau level corresponding to higher urea initial concentration). However, the time to reach this plateau depends on the reaction kinetics and thus mainly on temperature and urease concentration [14, 16] as seen on Figures 3b and 3c.

On the contrary, for water medium (Figure 6b), urea concentration stayed nearly unchanged when the “apparent” plateau of pH was reached at the end of the experiment (after 4 minutes). To explain the “apparent” plateau reached in water medium, a longer experiment (150 min)

was simulated for an initial urea concentration of 0.150 mol.L^{-1} , an initial $\text{pH} = 4.7$, and with a temperature of 310 K for an urease concentration of $3,500 \text{ U.L}^{-1}$ (in order to simulate faster kinetics). Results of this simulation (not shown here) confirmed that it is an “apparent” plateau since a low pH increase rate of 0.006 min^{-1} is maintained. Urea hydrolysis rate r_{hydro} was calculated in water and was reported in Figures 7a (*versus* the time) and 7b (*versus* the pH). For comparison, the same experiment was also simulated in 2.5% w/v chitosan medium and urea hydrolysis rate r_{hydro} in chitosan medium is also represented in on Figure 7.

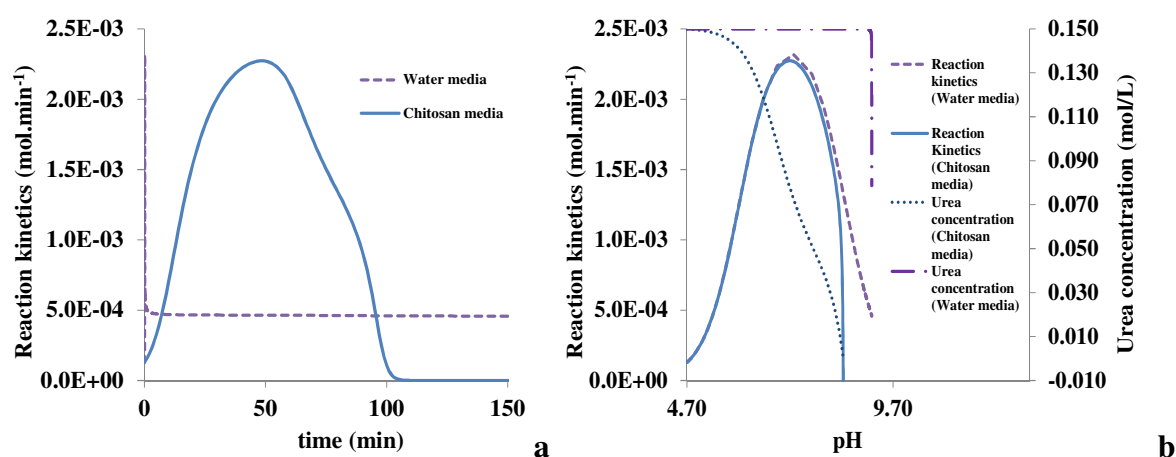


Figure 7: Variation of urea hydrolysis rate in water and 2.5% w/v chitosan media for $[(\text{NH}_2)_2\text{CO}]_0 = 0.150 \text{ mol.L}^{-1}$, $T = 310 \text{ K}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$: a) *versus* time and b) *versus* pH (with also the urea concentration)

As expected from the pH measurement in water medium (the fast increase of the pH at the beginning of the reaction), Figure 7a exhibits a peak of the hydrolysis reaction rate at $2.3 \times 10^{-3} \text{ mol.min}^{-1}$ at the beginning of the reaction (for time less than one minute) then it reaches a value below $5.0 \times 10^{-4} \text{ mol.min}^{-1}$ and exhibits a very slow decreasing. On the contrary, the hydrolysis rate has a bell-shape around a maximum value of $2.3 \times 10^{-3} \text{ mol.min}^{-1}$ in chitosan medium. Figure 7b shows variations of reaction rate (with also the variation of urea concentration) in water and chitosan media *versus* pH . The dependence of the hydrolysis rate on the pH is very similar up to a value of 7.2 which corresponds to the optimum pH for enzyme activity. Until this optimum, pH is reached (few seconds in water medium and about 50 minutes in chitosan medium), urea is still at a high concentration and the reaction limiting parameter is the low pH . After this optimum pH value, a large amount of urea quantity was consumed in chitosan medium because of longer experiment time corresponding to a concentration below 0.075 mol.L^{-1} : thus urea concentration becomes the limiting parameter

for the hydrolysis reaction rate and then the depletion of urea induces the final drop of the hydrolysis rate. On the contrary, the limiting parameter in water medium is always the pH since urea concentration is maintained high and never becomes the limiting parameter. The “apparent” plateau of pH reached in water medium is thus due to the influence of pH that slowdown the hydrolysis reaction at higher value but does not completely stop it. It is also important to note that the pH at the “apparent” plateau is close to the ammonia/ammonium equilibrium ($pK_a = 9.3$) which also contributes to slow-down the pH increasing by a buffer effect. Finally, it is interesting to point out that the surprising trend of the curve representing the urea concentration *versus* pH is easily explained by the fact that only few urea is consumed to reach a pH of 9.2 (very short experiment duration) and then urea concentration decreases for a longer period of time (at a rate of around $0.464 \times 10^{-3} \text{ mol.L}^{-1}.\text{min}^{-1}$) while the pH exhibits a very slow variation (at a rate of only 0.006 min^{-1}) during this same period.

To conclude this section, pH measurement can be a useful tool for monitoring urea enzymatic hydrolysis reaction rate in water medium, but only for the initial time of the reaction before the “apparent” plateau, while pH measurement might characterize the global chitosan enzymatic gelation rate in chitosan medium.

4.4 Monitoring of gelation and urea hydrolysis reaction kinetics in chitosan medium

Figure 8a presents the pH time-variation of the simulated experiment in 2.5% w/v chitosan medium for an initial urea concentration of 0.150 mol.L^{-1} , an initial pH 4.7, a temperature of 310 K and an urease concentration of $3,500 \text{ U.L}^{-1}$.

Gelation point is usually defined thanks to rheological measurements [25] when the elastic modulus G' (storage modulus which represents the gel solid component) overcomes the viscous modulus G'' (loss modulus which represents the gel liquid component). It corresponds to the formation of inter-molecular non-covalent bonds (hydrogen bonds and van der Waals interactions) because of the deprotonation of chitosan amino-groups. At this gelation point, a gelation time t_g and a corresponding gelation pH_g can be defined [16]. However, at this pH, gel can still flow and in previous study, we defined an advanced gelation time t_{ag} with a corresponding advanced gelation pH_{ag} where a fully rigid gel (that cannot flow anymore) was obtained.

A complementary approach can be to consider gelation as the chemical equilibrium between

the protonated $\text{Chit} - \text{NH}_3^+$ and non-protonated $\text{Chit} - \text{NH}_2$ forms of the amino-groups of the chitosan *D*-glucosamine unit. According to this definition, a gelation degree may thus be defined according to Equation 24 and is also shown on Figure 8a (purple dashed line). This gelation degree indicates the ratio of chitosan non-protonated *D*-glucosamine units over the total chitosan *D*-glucosamine units (both protonated and non-protonated).

$$\text{Gelation} = \frac{[\text{Chit} - \text{NH}_2]}{[\text{Chit} - \text{NH}_3^+] + [\text{Chit} - \text{NH}_2]} \quad \text{Equation 24}$$

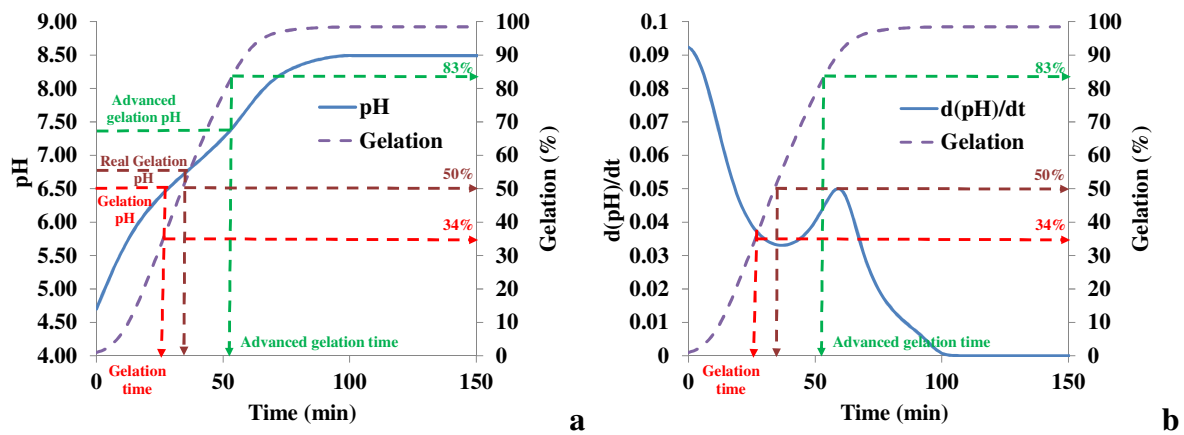


Figure 8: Variations of gelation percentage versus time for $[(\text{NH}_2)_2\text{CO}]_0 = 0.150 \text{ mol.L}^{-1}$, $T = 310 \text{ K}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$ and a non-buffered 2.5% w/v chitosan solution a) with the pH variations and b) with the dpH/dt variations.

Figure 8a shows that if a gelation pH_g of 6.5 is considered as in our previous work [16] for a time of 27 minutes, it corresponds to a gelation degree of only 34%. Figure 8b represents the time-variation of $d(\text{pH})/dt$. The local minimum of this curve is obtained at 32 minutes which corresponds to pH of 6.7, *i.e.* the pK_a of the chitosan amino-groups retained for the modeling. Indeed, the slowest variations of pH obtained in the buffer zone of the chitosan amino-groups. For the simulations, this pH must thus be considered the “real gelation pH” and correspond to a gelation degree of 50%.

Gelation pH can thus be defined both as the pH when G' and G'' modulus are equal and as the pH equal to the pK_a of the chitosan amino-groups, but it must be noted that both definitions only concern the “initiation” of the gelation process that is a continuous evolution: if a gel is well obtained, it does not signify that the gel is rigid and does not flow. Moreover, of only little difference is obtained between the gelation times if gelation pH of 6.5 (27 minutes) or 6.7 (32 minutes) are considered, this small variation of pH in this zone induces an important

variation of the gelation degree.

For the advanced gelation time (when a rigid gel is obtained) [16], a gelation degree of 83% was obtained which reports on a higher gel rigidity than at the gelation time thanks to the presence of a larger percentage of chitosan non-protonated *D-glucosamine* units. This point could be considered the end of the gelation process.

Following $d(\text{pH})/dt$ as a function of time seems to be a good way for determining the gelation pH. In order to validate this method, Figure 9 presents variations of $d(\text{pH})/dt$ for the experiment corresponding to 2.5% w/v chitosan medium, 0.150 mol.L⁻¹ urea concentration, 298 K and a 3,500 U.L⁻¹ urease concentration (experiment of Figure 3a).

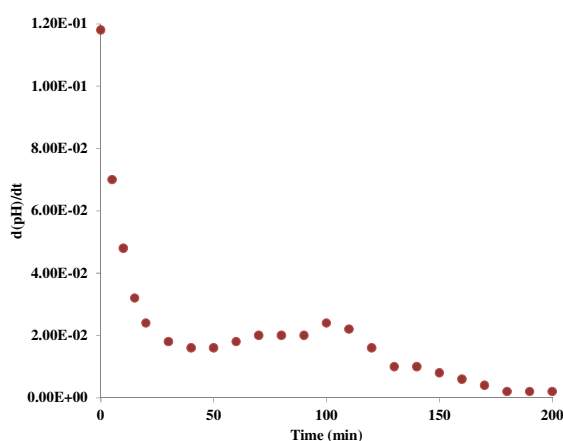


Figure 9: Variations of dpH/dt for for $[(NH_2)_2CO]_0 = 0.150 \text{ mol.L}^{-1}$, $T = 298 \text{ K}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$ and a non-buffered 2.5% w/v chitosan solution

The local minimum of the curve was obtained at a time 45-50 min for a corresponding pH of around 6.52 - 6.69. These values can thus be defined as the gelation time and gelation pH, respectively, for this experiment. It corresponds to the value determined by rheological measurements of $\text{pH} = 6.5$ which also thus correspond to the experimental pK_a value of the amino-groups of the particular chitosan used in these experiments. It can be noted that it slightly differs from the mean pK_a value of the chitosan amino-groups ($\text{pK}_a = 6.7$) that was retained for the modeling. A strong and direct similarity between the mechanical property (rheological measurement of the gelation point) and the chitosan chemical equilibrium (measurement of pH) exists.

In chitosan medium, monitoring pH is thus a good way to measure the global enzymatic

gelation and determine the gelation time and the advanced gelation time without using rheological measurements. Furthermore, a thorough analysis of pH evolution can also give some useful information to differentiate the chitosan gelation kinetics and the enzymatic reaction kinetics.

Figure 10 presents time-variation of the chitosan gelation rate r_{gel} (defined according Equation 25), the urea hydrolysis reaction rate r_{hydro} for the same simulated experiment of Figure 8 and the variation of $d(pH)/dt$ variations *versus* time.

$$r_{gel} = \frac{d([Chit-NH_2])}{dt} \quad \text{Equation 25}$$

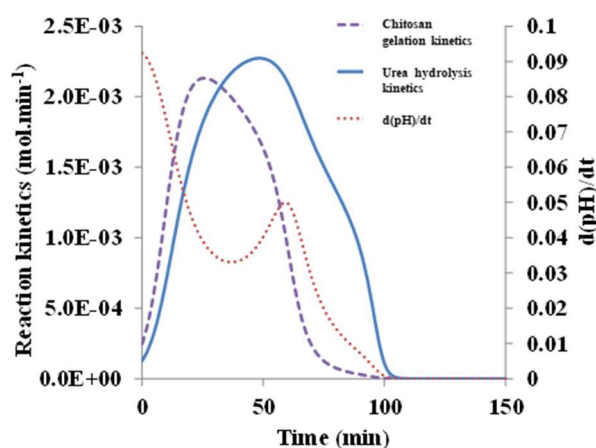


Figure 10: Variations of $d(pH)/dt$, chitosan gelation and urea hydrolysis reaction kinetics *versus* time for an urea concentration for $[(NH_2)_2CO]_0 = 0.150 \text{ mol.L}^{-1}$, $T = 310 \text{ K}$ and $[E]_0 = 3,500 \text{ U.L}^{-1}$ and a non-buffered 2.5% w/v chitosan solution

Figure 10 shows that the maximum rate of the chitosan gelation and urea hydrolysis reaction were not concomitant. Maximum rate for the chitosan gelation occurred around the gelation pH (*i.e.* the pK_a of the chitosan amino-group) whereas the maximum rate for the urea hydrolysis reaction occurred around the optimum pH for the urease activity (see Section 4.3). In chitosan medium too, pH is thus a good indicator for the chitosan gelation and urea hydrolysis kinetics. Indeed, the local minimum of $d(pH)/dt$ corresponds nearly to the maximum of chitosan gelation rate (Reaction 1) while the local maximum of $d(pH)/dt$ corresponds nearly to the maximum urea hydrolysis reaction rate (Reaction 2). It must be noted that those correspondences may not be exactly the same because of the presence of the other equilibrium reactions: ammonia/ammonium equilibrium and carbon dioxide/carbonate/hydrogen carbonate equilibrium.

5. Conclusion

Based on urea hydrolysis kinetics modeling in water medium, an enzymatic chitosan gelation kinetics modeling was developed and successfully validated by experimental measurement in batch stirred reactor. Comparison between urea hydrolysis reaction rates in water and chitosan media exhibited that the chitosan has nearly no influence on the enzymatic kinetics parameters. The differences observed in pH measurement were only due to the presence of the chitosan equilibrium.

Simulations results allowed decoupling and better understanding gelation and urea hydrolysis reaction and pointing out the limitation of urea hydrolysis reaction by urea concentration depletion in chitosan medium and by pH in water medium. Modeling results exhibited also that monitoring pH and $d(\text{pH})/dt$ was a good indicator for the determination of (i) the gelation time, (ii) the advanced gelation time, (iii) time of the maximum gelation rate and (iv) time of the maximum urea hydrolysis rate.

Next step of this modeling will be the addition of internal and external mass and heat transfer modeling in order to deeply investigate fate of dissolved organic carbon and ammonia that can be present in the solution.

6. Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

7. List of Symbols

C_c	Chitosan mass concentration	% w/v
C_c'	Chitosan mass concentration	g.L^{-1}
DDA	Chitosan degree of deacetylation	%
Ea	Arrhenius Equation activation energy	J.mol^{-1}
K_1	Chitosan non-protoned and protoned amino-group equilibrium constant	-
K_3	Ammonia and ammonium equilibrium constant	-
K_4	Carbonate and hydrogen carbonate equilibrium constant	-
K_5	Carbon dioxide and hydrogen carbonate equilibrium constant	-
$K_{ES,1}$	Enzyme-substrate complex 1 molecular dissociation constant	mol.L^{-1}

$K_{ES,2}$	Enzyme-substrate complex 2 molecular dissociation constant	mol.L ⁻¹
K_W	Water dissociation constant	-
K_M	Enzyme Michaelis constant	mol.L ⁻¹
K_P	Enzyme product (ammonium) inhibition constant	mol.L ⁻¹
K_S	Enzyme substrate (urea) inhibition constant	mol.L ⁻¹
k'	Enzyme activity-mass conversion factor	U.g ⁻¹
k_0	Arrhenius Equation pre-exponential factor	mol.U ⁻¹ .min ⁻¹
k_T	Temperature-dependent urea hydrolysis reaction rate coefficient	mol.U ⁻¹ .min ⁻¹
$k_{T,pH}$	Urea hydrolysis reaction rate coefficient	mol.U ⁻¹ .min ⁻¹
MW_{Chit}	Chitosan molecular weight	g.mol ⁻¹
MW_{Glc}	Chitosan unit mean molecular weight	g.mol ⁻¹
MW_{GlcN}	Chitosan <i>D-glucosamine</i> unit molecular weight	g.mol ⁻¹
MW_{GlcNAc}	Chitosan <i>N-acetyl-glucosamine</i> unit molecular weight	g.mol ⁻¹
N_{GlcN}	Number of moles of <i>D-glucosamine</i> units by mole of chitosan	mol.mol ⁻¹
pKa	pKa of the chitosan amino-groups	-
pH	pH	-
pH_0	Initial pH	-
pH_{ag}	Advanced gelation pH	-
pH_g	Gelation pH	-
R	Universal gas constant	J.mol ⁻¹ .K ⁻¹
r_{exp}	Experimental urea enzymatic hydrolysis reaction rate	mol.L ⁻¹ .min ⁻¹
r_{gel}	Chitosan gelation rate	mol.L ⁻¹ .min ⁻¹
r_{hydro}	Urea enzymatic hydrolysis reaction rate	mol.L ⁻¹ .min ⁻¹
T	Temperature	K
T^*	Temperature kinetics parameters for [18]	K
t	Time	min
t_{ag}	Advanced gelation time	min
t_g	Gelation time	min
$\alpha_{ES,1}$	Enzyme-substrate complex 1 empirical temperature-independent constant	-
$\alpha_{ES,2}$	Enzyme-substrate complex 2 empirical temperature-independent constant	-
v_{max}	Urea enzymatic hydrolysis maximum reaction rate	mol.L ⁻¹ .min ⁻¹
ϕ_{pH}	pH-dependent urea hydrolysis reaction rate coefficient	-

	correction factor	
$[Chit - N]_0$	Chitosan total amino-group initial concentration	mol.L ⁻¹
$(Chit - NH_2)$	Chitosan non-protoned amino-group activity	-
$[Chit - NH_2]$	Chitosan non-protoned amino-group concentration	mol.L ⁻¹
$(Chit - NH_3^+)$	Chitosan protoned amino-group activity	-
$[Chit - NH_3^+]$	Chitosan protoned amino-group concentration	mol.L ⁻¹
$[Cl^-]$	Chloride concentration	mol.L ⁻¹
(CO_2)	Carbon dioxide activity	-
$[CO_2]$	Carbon dioxide concentration	mol.L ⁻¹
(CO_3^{2-})	Carbonate activity	-
$[CO_3^{2-}]$	Carbonate concentration	mol.L ⁻¹
$[E]_0$	Urease concentration	U.L ⁻¹
(H_2O)	Water activity	-
(H_3O^+)	Hydronium activity	-
$[H_3O^+]$	Hydronium concentration	mol.L ⁻¹
(HCO_3^-)	Hydrogen carbonate activity	-
$[HCO_3^-]$	Hydrogen carbonate concentration	mol.L ⁻¹
$[(NH_2)_2CO]$	Urea concentration	mol.L ⁻¹
$[(NH_2)_2CO]_0$	Urea initial concentration	mol.L ⁻¹
(NH_3)	Ammonia activity	-
$[NH_3]$	Ammonia concentration	mol.L ⁻¹
(NH_4^+)	Ammonium activity	-
$[NH_4^+]$	Ammonium concentration	mol.L ⁻¹
(OH^-)	Hydroxide activity	-
$[OH^-]$	Hydroxide concentration	mol.L ⁻¹

8. References

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