

## Article

# Mathematical Modeling of the Kinetics of Glucose Production by Batch Enzymatic Hydrolysis from Algal Biomass

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**Abstract:** The processing of *Kappaphycus alvarezii* algae to obtain carrageenan (polysaccharide) generates a residue composed mainly of glucans and galactans that can be converted to monosaccharides, making these algae a renewable feedstock that can be used to produce biofuels. This residue was subjected to batch enzyme hydrolysis with different commercial enzymatic cocktails, achieving, after 72 h of reaction time, a complete conversion of glucan to glucose for all the cocktails used. A simple mathematical model, based on a semi-empirical approach, was proposed to describe the behavior of the experimental data. The temporal profile of glucose concentration was obtained by direct analytical integration of the mathematical model, resulting in an explicit equation as a time function. Estimation of the model parameters was carried out by non-linear regression, using the least squares criterion, together with the Levenberg–Marquardt method. The quality of the model fit was evaluated by specific statistical criteria, including Fisher’s *F* test, the  $R^2$  value, and the *p*-value test. The accuracy of the model was considered acceptable (*p*-value < 0.05 and  $R^2 \geq 0.98$ ), enabling its use in subsequent studies aimed at improving the enzymatic hydrolysis process under similar experimental conditions.



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**Keywords:** biomass conversion; enzymatic catalysis; kinetic modeling; parameter estimation; model fit

## 1. Introduction

In recent years, the production of biofuels has become a strategic issue due to economic and environmental aspects, justifying studies to find new raw materials, technologies and processes, aiming for greater profitability and sustainability in this sector.

Ethanol has been produced by fermentation processes using mainly sugar cane, corn starch, and lignocellulosic materials. Due to their complex chemical structures based on polymers, the latter materials require more severe processing conditions in the biomass pretreatment stage than the first ones to obtain quantitative conversions. Conversely, high severities lead to inhibitor formation, which are undesirable in the subsequent fermentation process [1].

Algal biomass is a potential raw material for biofuel production since these organisms present, in their composition, a high percentage of carbohydrates and a low percentage of lignin, which make the polymers contained in this biomass more accessible to enzymatic hydrolysis [1]. The production of third-generation biofuels is based on the use of algae, including *Kappaphycus alvarezii*, a seaweed of high industrial interest, used for carrageenan

production, which is mainly used in the food industry as a thickening, gelling, stabilizing, and suspending agent in water and milk systems [2].

A joint research and development effort into enzymatic hydrolysis processes has been undertaken due to their potential to quantitatively convert biomass to glucose [3]. Enzymatic hydrolysis of the residue generated during carrageenan production from the macroalgae *K. alvarezii* is a promising process to produce fermentable monomeric sugars (glucose) for biofuel production since the byproduct is composed mainly of a fraction of glucans easily hydrolyzed by commercial enzymes with cellulase activity, while the other fraction, based on galactans, is poorly hydrolyzable [1]. However, there is still a lack of technological development to reach cost-competitive productivities, thus justifying process optimization studies, based on mathematical models, to overcome the bottlenecks to economic and environmental feasibility [2].

Mathematical modeling aims to better understand the kinetic behavior of polysaccharide (polymer) hydrolysis in order to develop hydrolytic enzymatic processes that can achieve high yields in glucose and other fermenting monomeric sugars [3]. Polysaccharide hydrolysis reactions are very complex, involving a substrate that is generally in a solid phase and a biocatalyst (enzyme) in a liquid phase, thus comprising a heterogeneous catalytic system, which is affected by several factors of different natures [4]. According to Bansal et al. [5], these factors are related to the following aspects: (i) substrate properties: concentration (solid/liquid ratio), crystallinity, degree of polymerization, lignin content, accessibility, and reactivity; (ii) enzyme properties: load, deactivation, product inhibition, and acting synergism; (iii) hydrolysis conditions: time, temperature, pH, and mixing. The mechanism of hydrolysis reactions can have several sequential steps, including physical and chemical steps, which makes it difficult to apply a phenomenological approach to modeling the kinetics of these reactions [4,5]. This difficulty has encouraged the development of empirical models [4,5]. In addition to these, there are semi-empirical models, which incorporate both empirical and phenomenological aspects in their formulations and have intermediate complexity.

In the phenomenological approach, the main phenomena involved in the system are described by mass and energy balances, principles of chemical and enzymatic kinetics, expressions for reaction rates, and of mass and heat transfer inter- and intra-phase [6]. In the empirical approach, the system is assumed as a black box, and the output variables are empirically correlated with the input variables by means of functions, which may include polynomial, exponential, logarithmic, hyperbolic, and sigmoidal models, among other adjustment models. Other examples of empirical models are statistical ones, which are based on the design of experiments and on the theory of multiple linear regression, as well as neural network models which are grounded in artificial intelligence concepts and are commonly used to describe complex dynamic systems [6]. Although simpler, several studies using empirical models have highlighted limitations in their applicability to conditions different from those for which the models were developed [7].

Once the models have been developed, they can be used for several purposes [8]: (i) to mathematically describe the dynamic relationship between the main species involved in enzymatic hydrolysis processes; (ii) to compare alternative conversion processes for different biomass feedstocks; (iii) to make predictions to significantly reduce labor-intensive experiments; (iv) to economically evaluate different processes for obtaining sugars.

In order to describe the kinetics of biomass depolymerization reactions catalyzed by cellulases (endoglucanase, exoglucanase, and cellobiase), which are a mixture of enzymes that act synergistically to convert cellulose into hexoses (glucose, galactose, and mannose), a fundamental and deep understanding of the physical and chemical properties of the reacting substrate and its respective interacting enzyme is required, followed by a full

investigation on the reaction rate-limiting factors [7]. A limitation to the hydrolysis rate is enzymatic digestibility, which can be increased by a suitable pretreatment step to remove recalcitrant compounds present in the original raw material, increasing the accessibility of enzymes to cellulose. For this purpose, direct heating methods using steam or hot water with or without acid addition can be used. Another limiting factor is the inhibition of the enzyme caused by the reaction product itself [9].

Chemical kinetics dealing with the mechanism and rate of reactions is a relevant issue to be considered in the design, optimization, and control of biomass conversion processes. Classical chemical kinetics is based on the analysis of original experimental data on concentration over time to obtain some important reaction kinetic parameters such as reaction rate constant, activation energy, pre-exponential factor, and initial reaction rate. Kinetic studies are generally based on elementary reaction steps, active intermediates, and the pseudo-steady-state approximation. The reaction intermediates are monitored to understand and to propose the reaction mechanism in its main sequential steps. Chemical kinetics has been widely applied in several areas, especially in catalysis and reactor design, with applications in biomass conversion processes [10].

In the present study, a semi-empirical mathematical model was developed to describe the hydrolysis kinetics of glucans (polysaccharides) contained in the residue generated in the carrageenan production from the macroalgae *K. alvarezii*. By measuring the concentration of glucose (monomer) formed over the reaction time in a batch system, it was possible to estimate the model parameters for the different enzymatic cocktails and catalyst loads used.

## 2. Results and Discussion

The biomass of *K. alvarezii* treated with KOH and extracted with water yielded 26.6% insoluble residue. The chemical composition of this residue revealed that its main components were glucan (38.4%), galactan (32.7%), ashes (18.2%), sulfate groups (13.3%), and insoluble aromatics (0.13%). Enzymatic hydrolysis tests were conducted at 2% consistency, with the insoluble residue containing 38.4% glucan. The final glucose concentrations obtained in the hydrolysis tests were consistent with the total glucan content in the insoluble residue subjected to enzymatic conversion. After the reaction, a solid residue remained, likely composed of non-hydrolyzable materials such as ash, sulfate groups, and residual carbohydrates like galactan.

Table 1 presents the parameter estimates and statistics indicators for the kinetic model adjustments to the experimental data obtained for each enzymatic cocktail and enzyme load ( $E$ ) used in the hydrolysis tests. It is observed from Table 1 that the standard deviations of the parameters are small, showing that the parameter estimates are accurate.

**Table 1.** Parameter estimates and statistical indicators for the kinetic model adjustments.

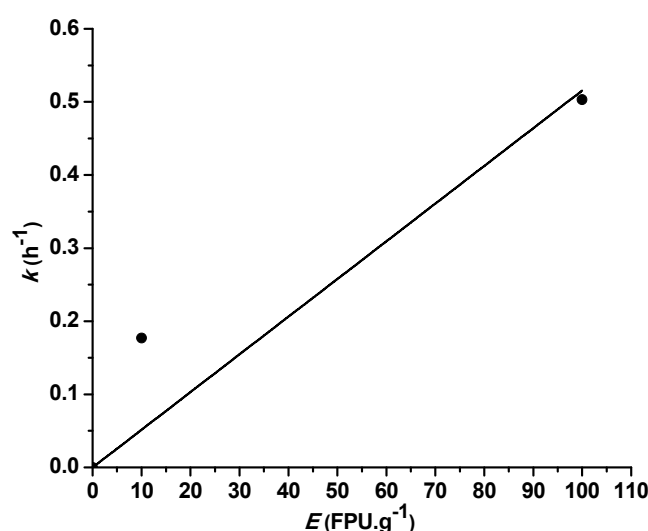
| Enzymatic Cocktail                      | $E$<br>(FPU.g <sup>-1</sup> ) | $C_{max}$<br>(g.L <sup>-1</sup> ) | $k$<br>(h <sup>-1</sup> ) | $\hat{k}$<br>(g.h <sup>-1</sup> .FPU <sup>-1</sup> ) | $r_{max}$<br>(g.L <sup>-1</sup> .h <sup>-1</sup> ) | $F_c$ | $R^2$ |
|-----------------------------------------|-------------------------------|-----------------------------------|---------------------------|------------------------------------------------------|----------------------------------------------------|-------|-------|
| Cellic CTec2                            | 10                            | 9.63 ± 0.38                       | 0.177 ± 0.028             | 0.0177                                               | 1.70                                               | 439   | 0.98  |
| Celluclast                              | 10                            | 9.44 ± 0.24                       | 0.0945 ± 0.0085           | 0.00945                                              | 0.89                                               | 1222  | 0.99  |
| Cellulase<br>from<br><i>Trichoderma</i> | 10                            | 8.48 ± 0.32                       | 0.0824 ± 0.0107           | 0.00824                                              | 0.70                                               | 596   | 0.99  |
| Cellic CTec2                            | 100                           | 8.94 ± 0.21                       | 0.503 ± 0.094             | 0.00503                                              | 4.50                                               | 1181  | 0.99  |

The value of the  $k$  parameter was around  $0.1 \text{ h}^{-1}$ , except for that estimated for the test performed with the enzymatic cocktail Cellic CTec2 ( $E = 100 \text{ FPU.g}^{-1}$ ), for which the value of  $k$  was  $0.5 \text{ h}^{-1}$ , a value about five times higher than those verified for the other cocktails. However, this high value of  $k$  is attributed to the high enzymatic load used in this test ( $E = 100 \text{ FPU.g}^{-1}$ ), providing the highest value for the kinetic constant ( $k$ ), which significantly increased the maximum reaction rate ( $r_{\max}$ ) to  $4.5 \text{ g.L}^{-1}.\text{h}^{-1}$ .

The importance of determining  $r_{\max}$  is that this parameter presents a parabolic behavior of descending concavity as the temperature increases, with a maximum located within the tested temperatures, this characteristic being very useful to find the optimal temperature for the hydrolysis reaction [11]. Although the effect of temperature on the hydrolysis kinetics has not been investigated in this study, the  $r_{\max}$  data obtained with both Cellic CTec2 enzyme cocktails show that this parameter was strongly dependent on the employed enzyme load. This effect would be the same for all cocktails, but for the Celic CTec2 one, it was clearer because two enzyme loads were used, one much larger than the other.

The  $C_{\max}$  parameter proved to be independent of the experimental conditions used, presenting small variations around its average value of  $9.12 \text{ g/L}$ , suggesting that the amount of cellulose available for hydrolysis is a given value in the biomass independent of the enzyme cocktail employed. This was an expected result since the  $C_{\max}$  parameter was estimated from the experimental data of glucose concentration obtained in hydrolysis tests using polysaccharide suspensions of equal consistencies ( $2\% w/v$ ), thus explaining the constancy of this parameter. This result indicates the need to use experimental data obtained under the broadest possible initial conditions, aiming to achieve the most representative estimate of the  $C_{\max}$  parameter. Assuming complete hydrolysis of glucan to glucose and using a stoichiometric yield factor of  $1.11 \text{ g/g}$ , the theoretical maximum glucose concentration would be  $8.53 \text{ g/L}$ . Therefore, the  $C_{\max}$  values slightly overestimate the maximum glucose concentration that could be achieved.

Since the relationship between  $k$  and  $E$  is linear ( $k = \hat{k}E$ ), where  $\hat{k}$  is the slope and  $0$  is the intercept, a fixed value for  $\hat{k}$  can be estimated from a linear regression of  $k$  versus  $E$ , using kinetic data referring to the same enzymatic cocktail (Cellic CTec2), in addition to the  $(0, 0)$  point (Figure 1). The value of  $\hat{k}$  thus estimated was  $0.00516 \text{ g.h}^{-1}.\text{FPU}^{-1}$ , and the coefficient of determination for the linear adjustment was  $R^2 = 0.92$ .



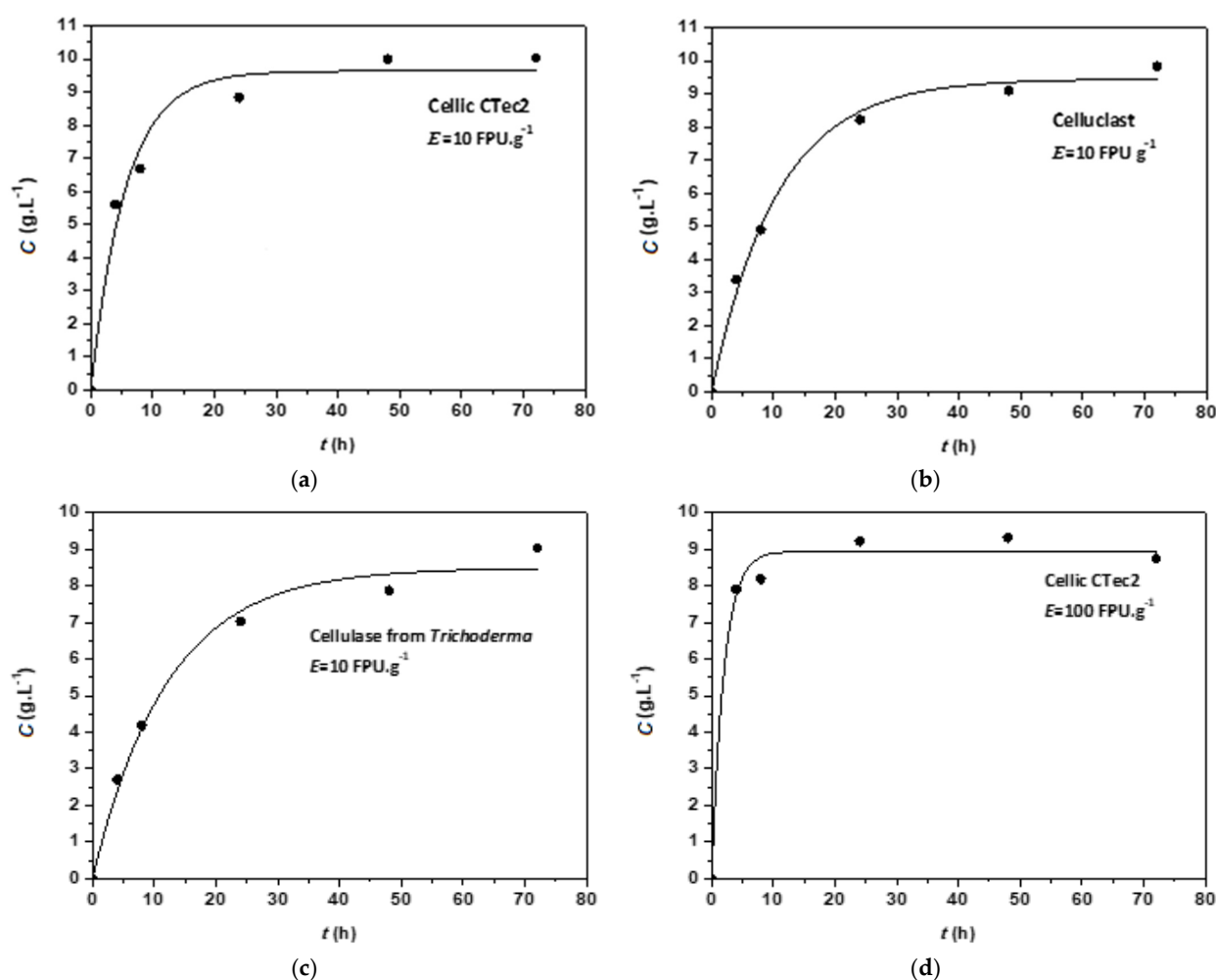
**Figure 1.** Linear regression to estimate the intrinsic kinetic constant ( $\hat{k}$ ) for the Cellic CTec cocktail.

It is very difficult to compare the parameter values obtained in this study with others reported in the literature, firstly because the models are generally different in their formu-

lations, with the respective parameters having their own meanings. Secondly, when the models are the same, the experimental conditions are generally very different, which can affect the values of some model parameters, making it difficult to conduct a reliable comparison between values of corresponding parameters. Despite these limitations, it was possible to verify that the  $k$  values obtained in this study are of a similar order of magnitude to those estimated by other authors [11,12] in kinetic studies, on the same enzymatic reaction in winemaking processes, using the same model outlined here. However, for the other model parameters ( $C_{max}$  and  $r_{max}$ ), it was not possible to make comparisons due to the initial conditions being very different, affecting the  $C_{max}$  and  $r_{max}$  values.

For validation purposes, the model predictions need to be confirmed with experimental data not used in parameter estimation [6]. When such experimental data are not available, an alternative way to assess the quality of the fit model is through appropriate statistical criteria. According to the adopted statistical criteria, the proposed kinetic model was validated for all hydrolysis tests, presenting indicators very favorable to its validation at a probability level greater than 95% (the  $p$ -value was the same for all the adjustments, i.e.,  $p < 0.05$ ). Furthermore, the  $R^2$  values were high ( $R^2 \geq 0.98$ ), indicating a good explanation capability of the mathematical model.

Figure 2 shows the experimental (points) and predicted (curves) temporal profiles of glucose concentration for all the performed hydrolysis tests. In general, the model predictions agree well with the experimental data.



**Figure 2.** Temporal profiles of glucose concentration in the enzymatic hydrolysis of glucan: (a) Cellic CTec2 cocktail ( $E = 10 \text{ FPU.g}^{-1}$ ); (b) Celluclast cocktail ( $E = 10 \text{ FPU.g}^{-1}$ ); (c) Cellulase from *Trichoderma* cocktail ( $E = 10 \text{ FPU.g}^{-1}$ ); (d) Cellic CTec2 cocktail ( $E = 100 \text{ FPU.g}^{-1}$ ).

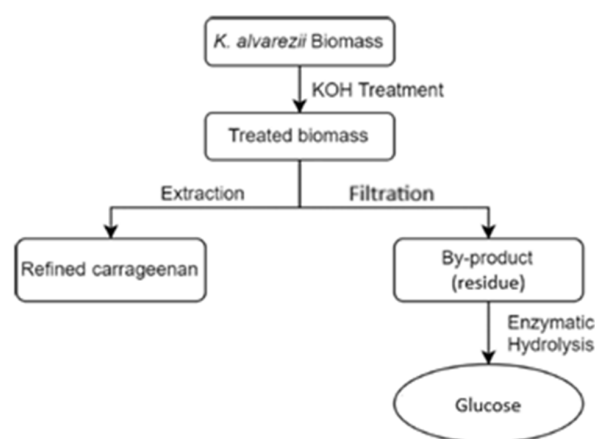
Although the proposed model provided good adjustments to the experimental data, it is necessary to highlight some important aspects, as follows: Model parameter estimation was based on a very limited number of experiments; i.e., only four experiments were carried out, one for each different enzymatic cocktail, at given conditions, and no other experimental factors were investigated, such as the solid/liquid ratio, temperature, agitation, etc. Moreover, model adjustment was performed for each experiment separately, providing two parameters for each cocktail, based on six glucose concentrations measured over 72 h. Good fitting of six points with a mathematical model having two parameters at only one condition does not indicate that the model would be applicable under other conditions, making it limited for extrapolation purposes. For example, the ideal temperature may not be the same for different enzyme cocktails, which requires careful investigation of this factor. At least, a small range of temperatures should be explored since the optimum temperature of cocktails may vary from 45 to 55 °C. A broader range of conditions, such as a higher solid/liquid ratio or the same ratio but starting from different solid masses, would be highly desirable to extend the possibility of extrapolating the model for use under other conditions at industrial scale.

### 3. Materials and Methods

#### 3.1. Experimental Procedure

The biomass of *K. alvarezii* was treated with a 6% KOH solution for 24 h at room temperature. The resulting material was washed, dried, milled, and extracted with water at 65 °C for 2 h. The suspension was filtered, resulting in an insoluble residue retained on the filter, while the fraction that passed through was identified as refined carrageenan. The chemical composition of the insoluble residue was assessed following NREL procedures for biomass composition [13].

The insoluble residue was hydrolyzed using four commercial cocktails of cellulases, identified as follows: Cellic CTec 2, Celluclast, and Cellulase from *Trichoderma*. All hydrolysis tests were conducted in 50 mL Falcon tubes at a consistency of 2% (*w/v*) in 50 mM sodium acetate buffer, pH 4.8, under rotary stirring at 120 rpm, a temperature of 45 °C, and a reaction time of 72 h. Samples from the reaction medium were collected at 4, 8, 24, 48, and 72 h to determine glucose concentration using HPLC (High-Performance Liquid Chromatography). Figure 3 presents a flowchart for obtaining the byproduct (residue) and its enzymatic hydrolysis to glucose. All hydrolysis tests were performed in triplicate, and the mean values and standard deviations of glucose concentration measurements were calculated for each time, obtaining an average experimental error of  $\pm 5.5\%$  of the measured value.

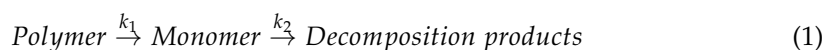


**Figure 3.** Process for obtaining the byproduct (residue) and sequential enzymatic hydrolysis for glucose production from residual glucan.

### 3.2. Model Development

In developing kinetic models for cellulose hydrolysis, some of the following key descriptions can be included [8]: (i) rate of decrease in cellulose concentration as a function of substrate and enzyme concentrations; (ii) rate of increase in glucose and/or soluble cellodextrin concentrations; (iii) product inhibition; (iv) enzyme binding; (v) temperature dependence; (vi) other factors.

The models proposed in the literature for hydrolytic reaction systems start from a pseudo-homogeneous approach, describing the hydrolysis process as a sequence of irreversible first-order reactions, schematically represented by the following:



The second reaction of the series is relevant in more severe processes such as acid hydrolysis. However, in enzymatic hydrolysis processes, due to the specificity of the catalyst, such a reaction is not observed to an appreciable extent, allowing it to be neglected in these cases. Thus, for a simplified mathematical modeling of the reaction kinetics under study, one starts from the scheme shown in Equation (1) and neglects the second reaction, as follows:



By performing a mass balance of glucose in the reaction medium in a batch system, Equation (3) is obtained, in which  $r$  is the glucose production rate in g/(L.h) units, evaluated by the variation in glucose concentration ( $C$ ) during given time intervals:

$$\frac{dC}{dt} = r \quad (3)$$

The rate of enzymatic reactions is generally given as a function of substrate concentration and enzyme load, according to the Michaelis–Menten equation, which is based on a reaction mechanism. However, the high difficulty in measuring substrate concentration in such real heterogeneous reactional systems has led to the use of alternative approaches for modeling the reaction kinetics. Zinnai et al. [12] modeled the kinetics of hydrolysis of  $\beta$ -glucans with  $\beta$ -glucanases by relating the reaction rate directly to glucose concentration. The equation proposed by the cited authors has a semi-empirical nature and was developed from the direct inspection of experimental data. As the behavior trend of experimental glucose concentration data observed in the present study was similar to that reported by Zinnai et al. [12], the kinetic equation proposed by them will be used here to model the kinetics of the reaction. Such a kinetic equation is first-order with respect to the difference between the maximum attainable glucose concentration ( $C_{max}$ ) and that in the time  $t$  ( $C$ ), i.e.,  $r = k(C_{max} - C)$ . The term  $(C_{max} - C)$  can be interpreted as the driving force of the reaction; i.e., the reaction proceeds as long as  $C$  does not reach its maximum value  $C_{max}$ . Thus, the reaction rate ( $r$ ) starts from its maximum value at  $t = 0$ , when  $C = 0$ ,  $r_{max} = kC_{max}$ , and gradually decreases until  $r = 0$  as the product (glucose) is accumulated in the medium. With this proposal, the experimental behavior of glucose concentration is expected to be adequately described, as it tends towards an asymptotic maximum value for long reaction times. Thus, by introducing the proposed kinetic expression for  $r$  in Equation (3) and integrating, Equation (4) is obtained, which is the temporal profile of glucose concentration ( $C$ ):

$$C = C_{max} (1 - e^{-kt}) \quad (4)$$

An inhibition effect by glucose could be included in the conception of the mathematical model since data already reported in the literature show that this product inhibits the

cellulase activity in these hydrolytic reactions [14–16]. The purpose of the mathematical model is to account for the mass of the reactant (the polymer that can be hydrolyzed or the biomass in this case) that is still unconverted at different times. This is not necessarily due to an inhibition effect, since natural products are not pure reactants and may have components that cannot be hydrolyzed.

### 3.3. Parameter Estimation and Model Validation

Due to the parameters  $k$  and  $C_{max}$  being derived from a pseudo-homogeneous approach, they incorporate several intervening factors in the hydrolysis kinetics and, for this reason, were estimated for each test, aiming to take into account the variations resulting from the different reaction conditions used (cocktail nature and enzyme load).

The parameters  $k$  and  $C_{max}$  were estimated for each hydrolysis test by non-linear regression, minimizing the sum of the squares of the residuals between the experimental values and those calculated by the model, according to the algorithm of Levenberg–Marquardt [17]. Six data points were used for each model adjustment.

Since it is well known that, in catalytic reactions, the constant  $k$  incorporates the intrinsic constant  $\hat{k}$  as well as the enzyme load  $E$  used in each test, this constant can be decomposed into the product of these two factors, i.e.,  $k = \hat{k}E$ .

To validate the kinetic model, some specific statistical tests were applied, which are described as follows: a different Fisher's F test, based on the regression sum of squares and on the residual sum of squares, was applied [17]:

$$F_C = \frac{\sum_{i=1}^n \frac{\hat{y}_i^2}{p}}{\sum_{i=1}^n \frac{(y_i - \hat{y}_i)^2}{n - p}} \quad (5)$$

In Equation (5),  $y_i$  and  $\hat{y}_i$  are, respectively, the experimental and calculated values of glucose concentration at time  $i$ ,  $n$  is the number of experimental data ( $n = 6$ ), and  $p$  is the number of model parameters ( $p = 2$ ).

If the calculated value of  $F$  ( $F_c$ ) is greater than the corresponding tabulated value of  $F$  ( $F_t$ ) for degrees of freedom  $p$  and  $n - p$ , and the probability is  $1 - \alpha$  ( $F_t(p, n - p; 1 - \alpha)$ ), the regression is considered statistically significant, with 0.05 being the value commonly adopted for the significance level ( $\alpha$ ). For all the adjustments performed, the tabulated value of  $F$  is  $F_t(2, 4; 0.95) = 6.9443$ .

The model was also tested by the  $p$ -value test, according to which the hypothesis that the model is valid is accepted if this value is lower than the significance level ( $\alpha$ ) set for the test.

Moreover, the coefficient of determination ( $R^2$ ) was calculated for each adjustment as a means of providing a fair first indication of how much of the variance in the experimental data is explained by the model.

All calculations were performed using the OriginPro 8.0 software from OriginLab Corporation (Northampton, MA, USA).

## 4. Conclusions

A simple semi-empirical kinetic model was proposed and employed to interpret experimental data from the enzymatic hydrolysis of residual biomass for generating glucose.

The hydrolysis tests were performed using as the substrate the polysaccharide fraction contained in the residue generated during the carrageenan processing of algal biomass (*Kappaphycus alvarezii*) for monomeric sugar production. This residue, mainly composed of

glucan and galactan, was subjected to hydrolysis employing several commercial enzymatic cocktails containing different enzyme loads.

This study followed a logical sequence of model development, parameter estimation, and model validation, demonstrating the model's applicability and reliability within the limited experimental conditions in which it was conceived and tested.

The model developed is semi-empirical and does not contain means of accounting for the different effects that can appear in such complex experimental conditions as the one occurring in this enzymatic hydrolysis. Furthermore, the model can hardly be extrapolated to other conditions or solid and enzyme masses, since it was developed based on the specific conditions of one experiment.

The mathematical model incorporates the kinetic effects induced by the enzymes employed (cellulases) and has a reduced number of adjustable parameters ( $k$  and  $C_{max}$ ), simplifying its implementation while effectively capturing the enzymatic kinetics.

The proposed model provided adjustments to the experimental data, with good statistical indicators, thus supporting its use for optimization studies of the enzymatic hydrolysis process. For example, the model can be used to estimate its own parameters ( $k$  and  $C_{max}$ ) from experimental data, allowing the maximum reaction rate ( $r_{max}$ ) to be evaluated by the product of these two parameters ( $r_{max} = kC_{max}$ ), which in turn can be employed in kinetic studies to determine the optimal temperature for the reaction. Once the mathematical model is validated, such a procedure can be an alternative to approximation or graphical methods, which are commonly used to determine maximum reaction rates by the classical differential method of kinetic analysis.

The use of algal biomass remaining from another process to generate fermentable sugars is a good example of circular economy and is interesting as a sustainable process, particularly due to the precursor used (a type of algae widely used to produce carrageenan).

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